A study of intra-ring checking and xylogenesis in *Pinus radiata* D. Don

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Hema Nair

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WOOD

"A piece of wood is a wondrous thing?",
Many will often say.
And to this fact I don't object
in any single way.
They'll talk forever about its strength
And versatility-
They'll paint it, carve it and cut it;
Yes-They go on endlessly.
With it they will build many homes,
From the fibres, paper they'll make-
They'll burn it and they'll bend it-
Even art forms of wood they'll create.
Now, I appreciate technology
In every form and way,
And I'm thankful for the progress
It gives to life each day.
But I desire to go outdoors
And view an ancient glade,
And sit and stare at greenish guards
That make the forests shade.
For in my heart I treasure wood
As it's naturally found to be:
Unheralded and priceless-
Just holding up a tree!

-Mickey Sull

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ABSTRACT

Pinus radiata is the dominant species of the plantations forests in New Zealand. The forest industry in New Zealand is heavily dependant on it. However, *Pinus radiata* can develop wood quality flaw called 'intra-ring checking'. The checks or splits appear in wood during kiln drying and usually affect the earlywood region of the wood. It lowers value of appearance grade timber leading to huge economic loses for the forest industry. This thesis presents a study that was undertaken as a part of ongoing collaborative work that is being carried out to understand wood quality issues in *Pinus radiata*, with a vision of improving its wood quality. This study was a part of that effort and was conducted with an aim to gain an insight into intra-ring checking, and the process of xylogenesis in *Pinus radiata*.

The investigations for this study were carried out in two steps. The first step was to understand intra-ring checking. The location of intra-ring checking was determined by observing the checks using various microscopy techniques. Scanning electron microscopy confirmed that checking was as an intercell failure that usually occurs at the cml/S_1 boundary. A comparative study was also conducted to see if the checked wood had some inherent properties that made it more susceptible to checking. It was found that checking could be influenced by tracheid geometry and cell wall thickness. If the wood had large tracheids with thin walls, it was more likely to develop checks during drying. Lignin distribution in the cell wall layers was also seen to play an important role in checking. Lower lignin levels and disruption in the pattern of lignification of the cell wall layers increased the tendency of the wood to develop checks. Similarly, if the tracheids have larger pits then their tendency to check increases. Structural features that disrupt the uniformity of the interlocking pattern of the tracheid such as rays and resin canals could also play a role in checking. Checked wood tends to have more surface area occupied by ray tissue. However, resin canals do not seem to be directly involved in checking, though their arrangement could indicate disturbances during xylogenesis.

The second step was to understand the process of xylogenesis in *Pinus radiata* especially with respect to the influence of auxin and boron on it. Nutrient and organ culture methods were manipulated and successfully used to study xylogenesis. An exhaustive comparative study was carried out to observe and measure selected wood properties. Microscopy and image analysis revealed that auxin and boron changes in the medium led to the alterations in the cell division, expansion and lignification. However, the analysis of the measurements and the observations displayed complex 'between-tree' and 'within-culture variations'. Clear trends did not emerge from the analysis hence, a confident conclusion on the association between auxin, boron and lignification could not be drawn from this organ culture study.

The study has added to the knowledge about checking and wood properties associated with it. A new tool of organ culture had been established that can help future research on the process of xylogenesis in *Pinus radiata*.

Abbreviations

$	dollars
%	percent
°A	degree Armstrong
°C	degree Celsius
ANNOVA	analysis of variance
B-RG-II	boron-rhamnogalacturonan-II complex
CAD	cinnamyl alcohol dehydorgenase
CCD	closed circuit digital
cm	centimeter
cml	compound middle lamella
FESEM	field emission scanning electron microscopy
g	grams
Hz	hertz
IAA	indole acetic acid
ICP	inductively coupled plasma-mass spectrometry
ICP-OES	inductively coupled plasma-optical emission spectrometry
kg	kilogram
kPa	kilopascals
kV	kilovolts
L	litre
M	moles per litre
mg	milligram
mg/L	milligrams per litre
mL	millilitre
mM	millimoles per litre
mm	millimeter
NAA	1-naphthalene acetic acid
OMT	*O*-methyl transferase
PBS	phosphate buffer saline
Py-Gc-MS	pyrolysis gas chromatography and mass spectrometry
RE	radially expanding cells
RG-I	rhamnogalacturonan-I

RG-II	rhamnogalacturonan-II
S_1	first secondary wall layer
S_2	second secondary wall layer
SEM	scanning electron microscopy
TEM	transmission electron microscopy
UV	ultraviolet
v/v	volume:volume ratio
w/v	weight:volume ratio
WQI Ltd	Wood Quality Initiative Limited
β	beta (anomeric configuration)
μM	micromoles per litre
μm	micron

Chapter One

Introduction and Literature Review

1.1 Thesis Aims

The forest industry in New Zealand is heavily dependant on *Pinus radiata* D. Don. The radiata pine wood is used as structural timber and in manufacture of various products like boards, veneers, panel products, pulp and paper. *Pinus radiata* has been grown in New Zealand for over a century, and is an important source of export revenues for the country. However, *Pinus radiata* wood during drying may develop a wood quality flaw called 'intra-ring checking'. The check lowers value of appearance grade timber leading to huge economic losses for the forest industry. The overall aim of this thesis was to gain an insight into intra-ring checking, and the process of xylogenesis in *Pinus radiata.*

The first part of the thesis concerns the investigations that were carried out to understand intra-ring checking. A better knowledge of the flaw could help in arresting the problem leading to good quality wood. The focus of the research was mainly to determine:

1) The site of intra-ring checking.

2) Differences between the wood that developed check on drying (checked wood) and wood that did not check (non-checked wood). A comparative study was conducted to see if the checked wood had some inherent properties that made it more susceptible to checking.

The major reason for the radiata pine plantations in New Zealand is to obtain timber for forest industry. Hence, it is important for the forest industry to harvest good quality wood free from any defects, to maximize their profits. Most tree improvement programmes so far emphasized selecting fast-growing, well-formed trees. However, now the New Zealand forest industry is focusing on improving wood quality properties related to specific end-product values. There is no short cut to improving wood quality. It is necessary to go to the source to gain more knowledge about the development of wood in *Pinus radiata* and the factors influencing it.

The second part of the thesis highlights the investigations conducted to understand xylogenesis in *Pinus radiata*. The steps taken in this direction were:

1) Establishment of organ culture of *Pinus radiata*. The organ cultures served as an ideal tool for carrying out experiments on the process of xylogenesis in *Pinus radiata*.

2) To probe the influence of the phytohormone auxin and the micronutrient boron on xylogenesis in *Pinus radiata* wood. The knowledge gained from growing wood in the controlled environment of a petri-dish could provide an insight to improving wood quality of *Pinus radiata* trees growing in New Zealand.

1.2 Plantation forest saviour of natural forest

Wood is an important renewable raw material (Tsoumis, 1991). It plays a key role in the global raw material market, with the forest industry as one of the world's most important industries. Forest industry represents close to 3% of the world's gross economic output (de Callejon & Lent, 1998). The value of the international trade in wood products reached US $ 150 billion in 2003 (International Tropical Timber Organization, ITT0, 2004). The overall demand for wood has been growing rapidly as the global picture of trade in wood products changed substantially with the emergence of China, the Russian Federation and Eastern Europe as major traders. We also see the traditional exporters of primary products in the Southeast Asia have been changing into exporters of secondary processed products because of the development of processing industries (Hashiramoto *et al.*, 2005). All this has increased the demand for wood that has led to the rapid logging of natural forests.

The timber industry is dependant on these forests, which are one of the critical ecosystems that are imperative for the health of the planet and for human well being (de Callejon& Lent, 1998). There is a growing concern about forest harvest levels worldwide. On a global average, more than one-third of all forests are primary forests or natural forests (defined as forests of native species where there are clearly no visible indications of human activities and where ecological processes are not significantly disturbed; FAO, 2005). There is a decrease in primary forest area due to deforestation, selective logging and other human interventions (FAO, 2005). There is a mounting pressure on the forest industry to reduce harvesting of natural forests, leading to stringent forest management standards (Hashiramoto *et al*., 2005). All these conditions have led to the development of plantation forests (a subset of planted forests defined as those consisting of primarily introduced species; FAO, 2005). The plantation forests are mainly established to meet some of the demand for wood and fiber from the timber industry, thus easing the pressure on the primary forests (FAO, 2005). *Pinus radiata* plantation forests have provided immense relief to the native forests of New Zealand and Chile where the native forests were being overcut. Chilean, pulp and paper industry relies heavily on the *Pinus radiata* stands (Scott, 1960). In 1997, 99% of New Zealand roundwood requirement was met by the *Pinus radiata* plantation forests (FAO, 2001, report). The success of the plantation forest has led to an increase in the area of the plantation forests worldwide. The area of plantation forests in the world has increased by 2.8 million hectares/year during 2000-2005 (FAO, 2005). In New Zealand 7% of the total land is under plantation forest (New Zealand Forest Owners Association, NZFOA, 2005/2006).

Global warming has become a major concern; hence, the role of the plantation forests is being appreciated not only as they supply timber for the forest industry, but also absorb carbon dioxide. In 2000, New Zealand's plantation forests absorbed 25 million tons of carbon dioxide (net of harvesting) (NZFOA, 2005/2006). If the present average new planting rate of more than 30,000 hectares/year is maintained, then it is estimated that over the period 2008-2012, New Zealand's plantation forests planted since the beginning of 1990, will remove more than 105 million tones of carbon dioxide from the atmosphere, worth as much as $2.5 billion in international carbon markets (NZFOA, 2005/2006). It is no wonder that some of the most important trees of the plantation forests

are the fast growing pines. They cover 20% of the plantation forests worldwide of which *Pinus radiata* is 4% (FAO, 2000).

1.3 *Pinus radiata,* an important plantation species of New Zealand plantation forests

The genus *Pinus* is classified in the plant kingdom as division Spermatophyta, sub division Gymnospermae, order Coniferae and family Pinaceae. Theophrastus established the genus identity, and it consists of over one hundred species (Mirov, 1967). Pines are conspicuous trees and man has used them for a long time not only as wood for fuel and building, but also as a source of fragrance for incense. The Aztecs, the Mayas, and the Romans used pine incense in religious ceremonies (Mirov, 1967). *Pinus radiata* D. Don (radiata pine, Monterey pine) evolved approximately 100 million years ago and is native to the coast of California (Millar, 1999). It also occurs as a two-needled variety on the Mexican island of Guadalupe (Scott, 1960; Mirov, 1967). In its own home radiata pine has negligible economic importance as it is overshadowed by the more plentiful Douglas fir and *Pinus ponderosa,* though it has been very successful exotic timber yielding tree in the southern hemisphere (Scott, 1960). The radiata pine was originally named Pinus insignis or 'remarkable pine'. It is an apt name for a tree that is able to grow in a range of growth conditions, easily propagated and yields timber in shorter time than many of the native species in both Australia and New Zealand (Scott, 1960; Johnston *et al*., 1997; Brooke & Langrish, 1997; McCurdy & Keey, 1999; NSW, 2006).

Radiata pine is the dominant species for the commercial forestry in New Zealand. It constitutes nearly 90 to 95% of the plantation forest resource (Mirov, 1967; Ministry of Agriculture and Forestry (MAF), 2005; Withers & Keena, 2001; Jayawickrama *et al*., 1997; Caughley & King, 2003). The high growth rate and large annual volume production are major factors responsible for the popularity of radiata pine (Scott, 1960; Kininmonth & Whitehouse, 1991). Radiata pine was a well established species in New Zealand by 1865, and has been part of the New Zealand plantation forest for over 100 years (Weston, 1957; Carson, 1987). New Zealand had 1,622,329 hectares of radiata pine planted by 2002, approximately 9% is older than 25years, 15% is aged 21-25 years, and

76% is less than 20 years old (MAF, 2005). The sawn timber exports are valued at NZ$ 920 million and the forest exports total $1 billion. The exports currently are up by 11% (NZFOA, 2005/ 2006). New Zealand forest industry supplies 1.1% of the world and 8.8% of Asia Pacific forest products trade, all from just 0.05% of the world's forest resource and an annual harvest equivalent to 0.0009% of global forest cover (NZFOA, 2005/ 2006). One of the main reasons for this is the highly productive, sustainably managed plantation forests of New Zealand (NZFOA, 2005/ 2006). The radiata pine plantation forests are important as they are making a major contribution to the forest industry, economy, environment of New Zealand and hence, chosen plant species for the study in this thesis.

Plantation trees	Hectares (thousands)	% of total
Radiata pine	1,626	89.2
Douglas- fir	112	6.2
Other exotic softwoods	33	1.8
All exotic hardwoods	51	2.8
Total	1,822	100

Table 1.1. *The plantation forest area by species in New Zealand as on 1st April 2004. The table clearly shows that radiata pine plantations occupy the maximum area of plantation forests in New Zealand (source, NZFOA, 2005/2006).*

1.4 Anatomy of gymnosperm wood with emphasis on radiata pine wood

Wood is a complex biological material known as secondary xylem (Butterfield & Meylan, 1980). Secondary xylem is generated from the vascular cambium and is found in the stems and roots of most gymnosperms and angiosperms (Butterfield *et al*., 1997). The vascular cambium is meristematic tissue that by repeated divisions not only maintains itself, but also forms radial files of secondary xylem on its inside, and secondary phloem on the outside (Chaffey, 2002a). This study concerns the secondary xylem or the wood, and will be discussed in detail further. The secondary phloem forms a protective outer

layer for wood. However, it is generally discarded while making wood products (Core *et al*, 1976), and it was not dealt with in the project investigations. The secondary xylem consists of mostly thickened cell walls deposited by the cytoplasm of each cell during differentiation. In most cells at functional maturity, the cytoplasm dies leaving them devoid of any living content, with only a hollow cell lumen with rigid cell walls (Esau, 1967; Thomas, 1977; Wilson & White, 1986; Tsoumis, 1991).

The wood of gymnosperms is simpler and more homogenous than that of angiosperms (Esau, 1967; Jane, 1970; Fahn, 1974; Meylan & Butterfield, 1972; Wilson & White, 1986). Gymnosperm wood is also referred to as *softwood*, a term derived from medieval timber trade (Butterfield & Meylan, 1980). Nearly 95% by weight and 90% by volume of the softwood is composed of long, fibrous tracheids arranged longitudinally in the stem (Mark, 1967; Panshin & de Zeeuw, 1970; Butterfield & Meylan, 1980; Grozdits & Ifju, 1984; Higuchi, 1997; Tsoumis, 1991; Hori *et al.*, 2002), and has only small amount of parenchyma particularly axial parenchyma (Jane, 1956; Butterfield & Meylan, 1980). Radiata pine is a gymnosperm tree, and displays the anatomical characteristics of softwood. Some of the main macroscopic and microscopic anatomical characteristics displayed by radiata pine are discussed in the sections that follow.

1.4.1 Macroscopic structural characteristics of softwood with emphasis on radiata pine wood

Structural features of radiata pine wood that are visible to the naked eye include the growth increments or growth rings (Figure. 1.1). In temperate zones, these growth rings are produced one per year and, hence, also referred to as annual rings (Harlow, 1970; Mauseth, 1995).

The first wood formed during the period of active growth is the earlywood while those cells that develop towards the end of the growing season form the latewood (Kozlowski & Pallardy, 1997; Figure. 1.1). The earlywood is considerably lighter in color than the latewood. This color separation is primarily due to differences in the structure of earlywood and latewood cells (Panshin & de Zeeuw, 1970; Thomas, 1991). The earlywood a has a high proportion of large lumened thin walled tracheids compared to smaller lumened, thick walled tracheids of latewood (Harlow, 1970; Butterfield &

Meylan, 1980; Core *et al*., 1976; Tsoumis, 1991; Mauseth, 1995). The thick-walled latewood tracheids provide strength, while thin-walled earlywood tracheids predominantly conduct water and minerals within the tree (Thomas, 1977; Fengel & Wegener, 1984).

Figure. 1.1 *The growth rings are clearly visible in oven-dried disc of radiata pine due to the differences in the earlywood and latewood. The earlywood band is considerably wider and lighter in colour than the latewood.*

1.4.2 Microscopic structural characteristics of softwood with emphasis on radiata pine wood

The two main cell types that constitute the wood of radiata pine are the tracheids and the wood rays. (Figure. 1.2). The tracheids are responsible for conduction of water and support while the parenchyma cells that constitute the wood rays store water and organic nutrients (Core *et al.*, 1976; Wangaard, 1981; Butterfield & Meylan, 1980; Higuchi, 1997; Kuhad *et al*., 1997; Climent *et al*., 1998; Barnett & Bonham, 2004).

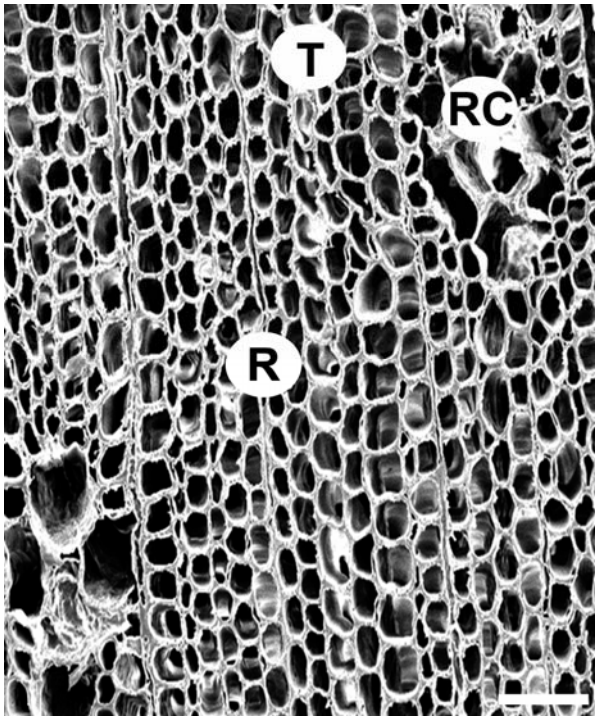


Figure. 1.2 *Scanning electron micrograph of the transverse section of Pinus radiata wood displaying the microscopic characteristic of the wood. The two main cell types that constitute the radiata pine wood are tracheids (**T**), and rays (**R**). The rays are usually uniseriate as shown in the micrograph. The rays may sometimes include resin canals (**RC**).Scale bar=10µm*

1.4.2.1 Tracheid

A tracheid is a cell, which on maturity develops a thick, lignified cell wall, with bordered pits (Wilson & White, 1986; Tsoumis, 1991; Barnett & Bonham, 2004). In its functional state, the tracheids in the wood are devoid of protoplast and comprise of only the non-living cell wall (Wilson & White, 1986; Tsoumis, 1991; Mittler & Lam, 1995). Tracheids originate from fusiform initials of the vascular cambium (Jane, 1970). During differentiation, they increase considerably in radial diameter but not tangentially, as a result they retain their alignment with the cambial cells from which they develop. Hence, the tracheids in softwoods, seen in transverse section, tend to be in rather regular radial files (Jane, 1970; Wilson & White, 1986; Butterfield & Meylan, 1980). The cells of mature wood are relatively long approximately 3-4 mm (Scott, 1960; Panshin & de

Zeeuw, 1970; Bamber, 1985). The tracheid is well suited to perform the functions of conduction and support due to the orientation of the long axis of the tracheid parallel to the long axis of the stem; this facilitates a longer passageway during water translocation prior to interruption by the cell wall, while the rigid cell wall provides adequate support (Thomas, 1977).

1.4.2.2 Bordered pits

Pits are areas through which sap may pass from one cell to another and usually occur in complementary pairs in adjacent walls, that is, two pits are combined into pair structures forming pit pairs (Esau, 1967; Meylan & Butterfield, 1972). Each half of the pit pair is separated by the pit membrane formed from the middle lamella and primary walls of the two cells (Meylan & Butterfield, 1972). There are two principal types of pits recognized in cells with secondary walls; these are *simple pits* and *bordered pits* (Esau, 1967; Meylan & Butterfield, 1972). The bordered pits in adjacent cell walls pair and join the neighbouring tracheids in conifers (Esau, 1967; Jane, 1970; Fahn, 1974; Core *et al*., 1976; Butterfield & Meylan, 1980; Wilson & White, 1986; Higuchi, 1997; Kuhad *et al*., 1997). The tracheids of the latewood do not have bordered pits. They have instead pits that tend to be smaller with elliptical or oval shape, and usually lack the raised borders that are seen in the earlywood tracheids (Panshin & de Zeeuw, 1970; Butterfield & Meylan, 1980).

The pits are usually circular in surface view, with a small circular opening called *pit aperture* that is covered by a *pit membrane* (Core *et al.*, 1976; Thomas, 1981). The pit membrane is not of equal thickness throughout; thus, a thickened circular area called the *torus* occupies the centre (Esau, 1967; Jane, 1970; Harlow, 1970; Fahn, 1974; Core *et al.*, 1976; Butterfield & Meylan, 1980; Wilson& White, 1986; Higuchi, 1991). This entire structure acts like a valve. When the torus is in central position of the pit chamber, the sap may pass from one cell to the next, however, if the torus is deflected against either aperture of the pit pair, it acts as a valve, flow of sap is obstructed, and the pit is said to be aspirated (Meylan & Butterfield, 1972, Booker, 2004).

1.4.2.3 Wood rays

The transversely oriented parenchyma cells occupying about 10% of the volume of wood constitute the wood rays (Thomas, 1981). They are living (retain protoplasm) and serve as storage cells as well as providing a radial pathway from the cambium into the sapwood (Core *et al.*, 1976; Wangaard, 1981; Tsoumis, 1991; Higuchi, 1997). The wood rays of radiata pine are composed of parenchyma cells and ray tracheids (Esau, 1967; Meylan & Butterfield, 1972). They develop from the ray initials of the cambium (Panshin & de Zeeuw, 1970). The ray cell wall may be primary or sometimes secondary as well (Meylan & Butterfield, 1972; Singh & Donaldson, 2000). The rays in radiata pine are mostly uniseriate (one cell thick, Figure. 1.2); except rays containing a resin canal are multiseriate near the canal, these are also referred to as *fusiform rays* (Butterfield & Meylan, 1980, Panshin & de Zeeuw, 1970).

In the upper or lower edges of the ray, or scattered among the ray parenchyma cells are ray tracheids, that either occur singly or in rows (Fahn, 1974), and are a common feature of the genus *Pinus* (Panshin & de Zeeuw, 1970). The cells of ray parenchyma are comparable to ray tracheids but are usually longer and have simple pits with no secondary wall thickenings (Panshin & de Zeeuw, 1970).

1.4.2.4 Resin canals

Longitudinal resin canals can be observed on the transverse surface and horizontal resin canals can be found incorporated into fusiform rays (Panshin & de Zeeuw, 1970; Jane, 1970; Core *et al*., 1976; Butterfield & Meylan, 1980). A resin canal is a complex of epithelial cells surrounding resin duct or cavity (Figure. 1.2; Panshin & de Zeeuw, 1970; Core *et al*., 1976; Wiendonhoeft & Miller, 2002). The resin ducts develop as intercellular spaces, by separation between resin producing parenchyma cells that form the epithelium of the duct (Esau, 1967; Fahn, 1974; Core *et al*., 1976; Wilson & White, 1986; Higuchi, 1997).

The wood may also show the presence of traumatic resin canals. The transverse canals like normal resin canals are confined to wood rays except that they are larger (Panshin & de Zeeuw, 1970).

1.5 Tracheid differentiation in conifers

The wood cell undergoes several changes during its formation, beginning with cell division in the cambium, followed by deposition of secondary walls, lignification and last the mortification of the cell protoplast (Fengel, 1970; Mittler & Lam, 1995). The changes that take place in the tracheid during its development can be studied *in vitro* as well as *in vivo*. Many *in vitro* studies have helped in understanding the process of tracheid differentiation as it happens in the plants (Leitch & Savidge, 2000).

1.5.1 Cambium

Growth in length of the stem or root is called primary or apical growth and it takes place by the activity of the apical meristem, while growth in diameter or secondary growth is achieved by the lateral meristem mainly the cambium (Tsoumis, 1991). Sanio (1863) distinguished the procambial cylinder from vascular cylinder, so the term cambium now signifies only lateral meristem (Philipson *et al*., 1971). In a growing wood, the group of cells depicting a similar stage of development have been grouped into zones. Figure. 1.3 gives an overview of the developmental zones in radiata pine growing stem. The zones that can be distinguished are: the zone of dividing cells (cambial zone, CZ), primary-walled radially expanding cells (RE zone), enlarged cells with developing bordered pits and producing secondary wall lamellae (SL zone) (Savidge, 2001).

The cambium consists of a one cell wide layer of initials and a small but varying number of layers of undifferentiated derivatives. However, the initials are difficult to distinguish from these layers. Hence, the whole complex is called cambial zone or cambial region (Larson, 1994). The cambium has two types of initials fusiform initials and ray initials (Philipson *et al*., 1971; Harris, 1991; Larson, 1994; Higuchi, 1997; Savidge, 2001). The majority are fusiform initials that produce all the axial cells of the xylem (Larson, 1994). The ray initials are narrow and short, slightly elongated to nearly isodiametric (Philipson *et al*., 1971; Tsoumis, 1991). The fusiform cells give rise to ray initials through anticlinal and/or successive asymmetric periclinal division (Savidge, 2001). Rays originate from ray initials (Phillipson *et al*., 1971; Higuchi, 1997).

Cambial initials divide periclinally, and produce xylem and phloem mother cells. These usually divide again, and their derivatives differentiate gradually into xylem and

phloem cells (Tsoumis, 1991; Larson, 1994; Higuchi, 1997). In radiata pine, the cambial initials divide periclinally twice or more to produce four daughter cells that undergo enlargement and differentiate further to form tracheids (Mahmood, 1968). The mechanism that stimulates activity of the cambial zone is not clear; however, it is widely accepted that stimulus comes from growth hormones mainly auxin (Zajaczkowski & Wodzicki, 1978; Wodzicki *et al.*, 1987; Savidge, 2001). Environmental factors and nutrient availability can also affect the cambial activity during a growing season (Tsoumis, 1991; Wodzicki, 2001).

1.5.2 The stages of tracheid differentiation

There are four stages that can be recognized in the development of the tracheids namely, stage of origin, enlargement, cell wall thickening and lignification (Wardrop, 1981; Harris, 1991; Hertzberg *et al*., 2001). The stage of origin involves the formation of fusiform and ray initials. The fusiform cells divide with one of the divided cells always remaining a fusiform initial, while the other cell is transformed into a xylem or phloem mother cell (Higuchi, 1997). The new cells so formed gradually enlarge towards their final size and shape increasing in diameter and length. The primary wall undergoes de-esterification of pectins through pectin methylesterase activity that releases protons and acidic polygalacturonans (Carpita & Gibeaut, 1993). The resulting drop in pH allows cell wall loosening and auxin induced growth. At the end of the enlargement stage, the new cell wall consists of solely primary wall. The deposition of the secondary wall by the protoplasm follows (Tsoumis, 1991). There are conflicting views about the initiation of secondary wall deposition. One view is that the secondary wall deposition begins after the cell stops expanding (Wardrop, 1964; Brett & Waldron, 1966) and the other is secondary wall deposition could be initiated before the cell stops expanding (Mühlethaler, 1965; Juniper *et al*., 1981; Abe *et al.*, 1997). As per Gritsch and Murphy (2005), the secondary wall began to be laid down while the cells were still undergoing some elongation, suggesting that it may cause the slow-down and eventual cessation of cell elongation. Secondary wall formation in radiata pine begins several cells in advance of lignification and appears to complete at about the same time the middle lamella is lignified (Donaldson, 1991).

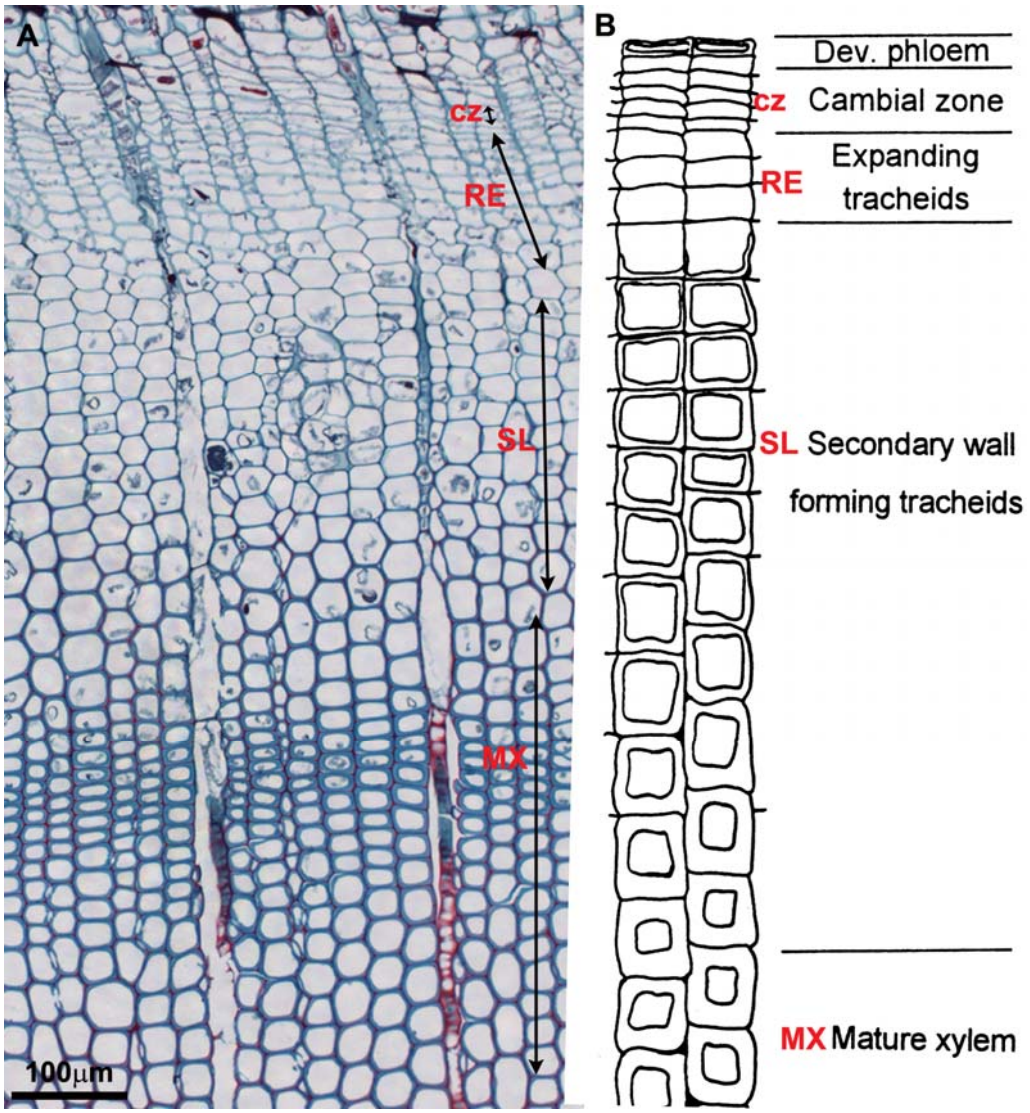


Figure. 1.3 *A) Transverse section of radiata pine wood showing the stages of development as followed in this thesis as per Savidge (2001). The zone of dividing cells (cambial zone,* ***CZ****), primary-walled radially expanding cells (****RE*** *zone), enlarged cells with developing bordered pits and producing secondary wall lamellae (****SL*** *zone or developing cells). B) Schematic drawing of transverse section to demarcate the zones more clearly (adapted from Uggla, 1999).*

Lignification is the final stage in the development of the tracheid cell, where lignin impregnates the cellulose and non-pectic polysaccharides network (Ye, 2002). Lignification was observed to occur first at the cell corners in the primary wall and middle lamella, gradual lignification then continues in the S_1 layer at the same time as the S_2 and S_3 layers are being formed (Wardrop, 1957; Forss & Fremer, 2003). In radiata pine lignification of the secondary wall does not begin until after the middle lamella has become fully lignified (Donaldson, 1991). Lignification in the middle lamella where carbohydrates have little or no orientation occurs more or less equally in all directions while in the secondary wall, where the cellulose is highly oriented, lignin deposition mostly occurs along the cellulose microfibril (Donaldson, 1994).

By the time lignification is complete the cells are devoid of protoplasm (Tsoumis, 1991). The warty layer when present develops during the final stage when lignification is complete or nearly so (Tsoumis, 1991). The stages of cell development are not clearly demarcated, deposition of secondary wall may start before the cell has reached its final size and shape, and likewise lignification may commence before deposition of secondary wall has ended (Savidge, 2001).

After completing the cellular activities necessary for the deposition of the secondary wall, developing tracheary elements undergo cell death and lose their cellular contents. Cell death initiation takes place by disruption of vacuole membranes, resulting in release of hydrolytic enzymes into cystosol (Groover *et al*., 1997; Groover *et al*., 1999; Kuriyama, 1999; Obara *et al*., 2001). This process could be similar to the formation of apoptotic bodies by animal cells during apoptosis (Mittler & Lam, 1995).

1.5.3 The wood ray differentiation

Since the rays in pine are uniseriate, the increase in the number of ray initials is not a result of divisions of existing ray initials, but addition of new ray initials (Esau, 1967; Panshin & de Zeeuw, 1970). New ray initials arise from fusiform initials by segmentation and this maintains a relative constancy in the ratio between the rays and axial components during the increase in circumference of the vascular cylinder (Esau, 1967; Larson, 1994; Savidge, 2001). Studies in conifers show that ray initiation is a complicated process involving the subdivision of fusiform initials, elimination of some

products of these divisions from the initial layer, and the transformation of others into ray initials (Esau, 1967; Larson, 1994). Rays may increase in width and height by fusion of two or more groups of ray initials (Esau, 1967; Larson, 1994). In the case of radiata pine, as with other conifers, ray initials differentiate into ray parenchyma and ray tracheids (Barnett, 1981).

Radial flow of developmental signals through ray initials in the cambial zone regulates the differentiation of the rays. Lev- Yadun & Aloni (1995) proposed a bidirectional radial signal flow hypothesis for regulation and differentiation of rays. According to their hypothesis, the radial flow of ethylene disturbs the axial flow of auxin and thereby limits the differentiation of tracheids. The axial signal controls the shape, direction and size of rays. The relationship between the axial and radial signal flows controls the orientation of cells within the rays.

1.6 The ultrastructure of softwood with emphasis on radiata pine wood cell wall

Electron microscopy observations show that wood cell walls are composed of an intercellular layer, a primary wall, and a secondary wall (Figure. 1.4; Barber & Meylan, 1964; Core *et al*, 1976; Butterfield & Meylan, 1980). The primary wall develops first then the secondary wall develops on the inside of the primary wall, usually after the elongation of the cell has ceased during later stages of cellular differentiation (Jane, 1970; Hori *et al*., 2002).

1.6.1 The compound middle lamella

The primary cell wall is a thin layer produced following cell division and during and subsequent growth of xylem mother cells (Buchannan *et al.*, 2002). The primary cell wall layer is 0.1 to 0.2 μm thick, with a random, interwoven network of cellulose microfibrils (Timell, 1965). An intercellular layer called the true middle lamella separates the neighbouring cells. As the primary walls of the two adjoining cells are difficult to distinguish from true middle lamella, the term compound middle lamella is used for the

three-ply layer that cements adjacent cells and appears as one layer between the secondary walls of the cells (Figure. 1.4; Fergus *et al*., 1969; Hafren *et al*., 2000).

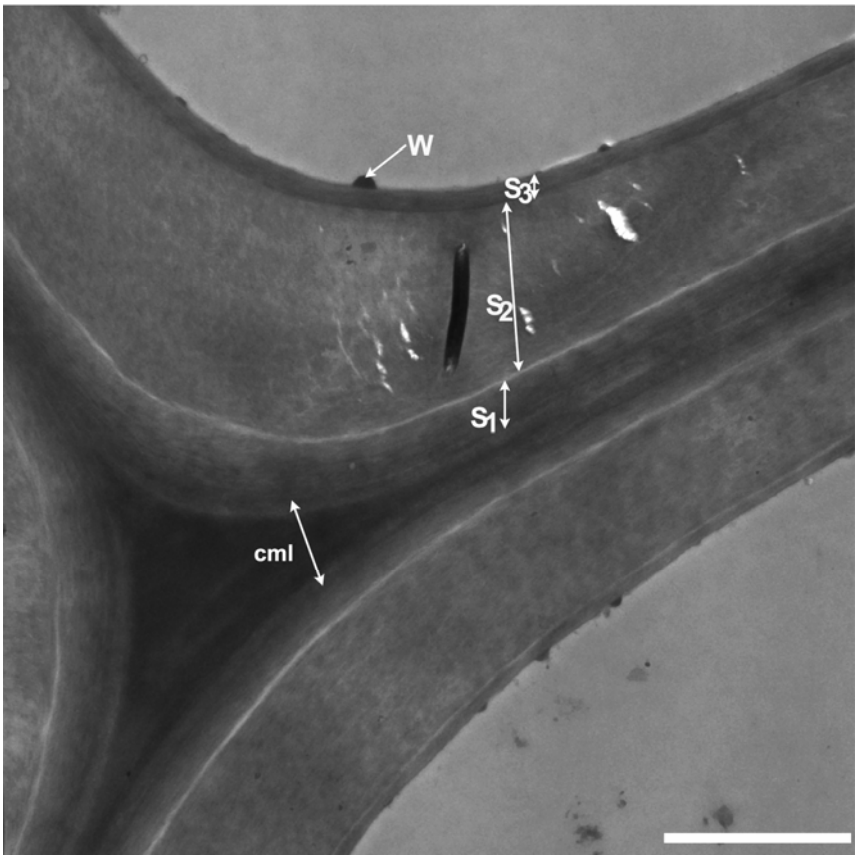


Figure. 1.4 *Electron micrograph of radiata pine wood cell wall layers. The compound middle lamellas (cml, is the darkly stained area) followed by the secondary wall layers the thin S_1 layer, followed by the bulky S_2 and thin S_3 layer followed by the innermost warty layer Scale bar=2µm.*

1.6.2 The secondary wall

The thickened, lignified *secondary cell wall* layer provides structural support independent of the turgor pressure of the cell (Esau, 1967; Fahn, 1974; Jarvis & McCann, 2000). The secondary cell wall consists of an outer layer closest to the primary wall

called S_1, a middle thick layer the S_2 layer, and the innermost layer adjacent to the lumen called the S_3 layer (as shown in fig 1.4a) (Core et al., 1976; Kuhad *et al*., 1997; Brandstrom, 2001).

The S_2 layer of the secondary cell wall layers is 1 to 5 µm thick, while S_1 is 0.1 to 0.3 µm thick, and the S_3 is 0.1 µm thick (Timell, 1965). The S_2 layer tends to dominate the physical and chemical properties of the cell wall (Donaldson & Burdon, 1995; Deresse *et al*., 2003; Huang *et al*., 2003; Donaldson & Frankland, 2004; Barnett & Bonham, 2004). The S_1 and S_3 wall layers do play a crucial role in strengthening cell wall against deformation by water tension forces, as well as contributing to transverse properties of cell wall (Booker & Sell, 1998; Bergander & Salmen, 2002; Donaldson & Frankland, 2004).

1.6.3 The characteristic organization of cellulose microfibrils in the cell wall layers

The cell wall layers can be recognized easily as they have a characteristic orientation of cellulose microfibrils (Donaldson & Singh, 1998; Hori *et al*., 2002; Huang *et al*., 2003; Barnett & Bonham, 2004). Figure. 1.5 shows the schematic diagram of the arrangement of the cellulose microfibrils with respect to the various cell wall layers. Within the primary wall, the microfibrils are loosely packed and arranged randomly without any lamellation (Barnett & Bonham, 2004). However, the microfibrils in the secondary wall are closely packed, and show a degree of parallelism. The average microfibril angle values seen in the three secondary wall layers with respect to the longitudinal axis are: the average angle of 50 to 70° for S_1, 10 to 30° S_2, and 60 to 90° for the S_3 layer. Usually transition lamellae between layers bring about gradual change in microfibril angles from layer to layer (Butterfield & Meylan, 1980; Wilson & White, 1986; Brandstrom *et al*., 2003; Barnett & Bonham, 2004).

In addition, a so-called *warty layer* is an integral part of S_3, forming an innermost wall layer (Butterfield & Meylan, 1980; Fengel & Wegener, 1984; Higuchi, 1997).
This layer is so called as it displays small protrusions that are laid down just prior to the death of the protoplast and covers the entire S_3 layer, pit cavities and any other wall sculpturing (Liese, 1965; Jane, 1970; Meylan & Butterfield, 1972; Higuchi, 1997).

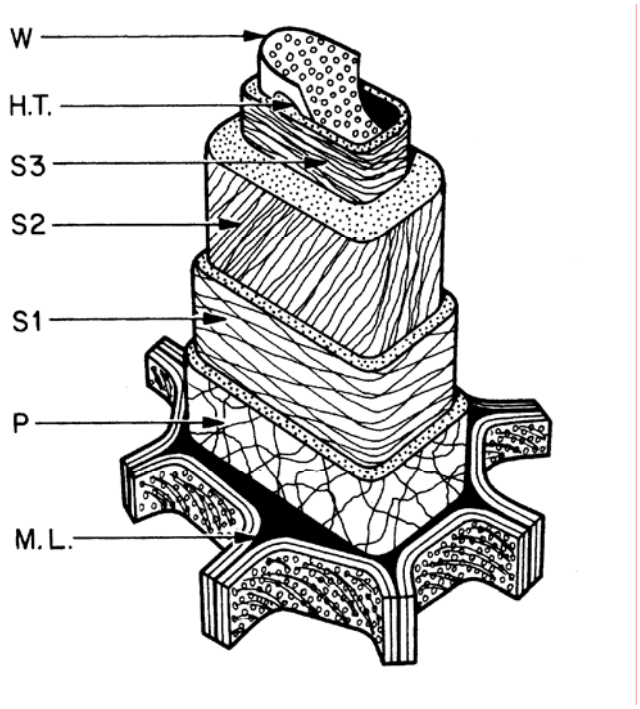


Figure. 1.5 *Schematic diagram to illustrate the general structure of a tracheid cell wall and the orientation of the cellulose microfibrils within each cell wall layer. (Butterfield & Meylan, 1980)*

1.7 The chemical composition of softwood with emphasis on radiata pine wood

Wood is a very complex and stable material. Various chemical substances that compose wood are intimately associated in the walls of wood cells (Figure. 1.6). Consequently, the methods of chemical analysis aimed at separating these substances, may also tend to degrade them and there is a possibility of their true nature being lost (Jane, 1970; Wilson & White, 1981). There is lack of consensus in the literature whether laboratory preparations are quantitatively and qualitatively representative of components, as they exist in wood in its natural state (Tsoumis, 1991). Chemical constituents of wood are classified into major components consisting of cell wall materials, and minor amounts

of extractives. The components of cell wall and intercellular layers amount to 90-95% of oven dry wood. Extractives occur mainly in cell lumen and special tissues such as resin cells (Higuchi, 1997).

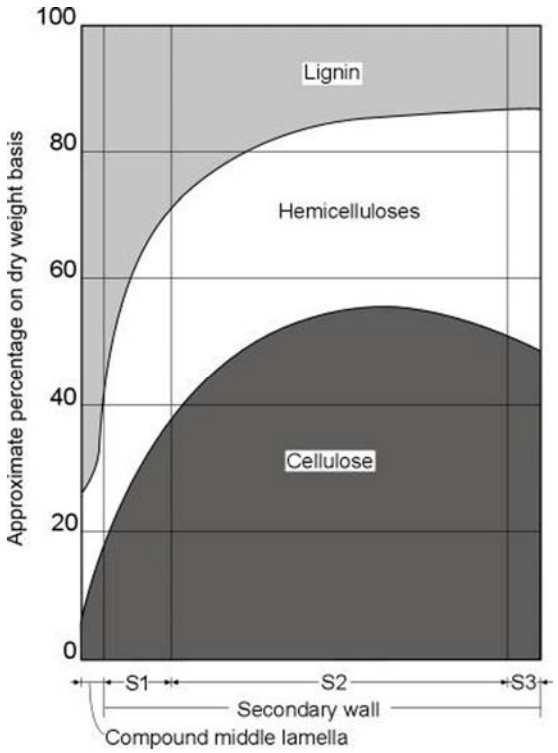


Figure. 1.6 *Schematic distribution of the principal chemical components within the various layers of the cell wall in conifers (Panshin & de Zeeuw, 1970). The primary wall has less than 10% cellulose. In the S_2 layer cellulose increases to more than 50% of the cell wall material and reduces again in the S_3 layer.*

1.7.1 Cellulose

Cellulose is the principal component of the cell wall (Carpita & Gibeaut, 1993; Buchannan *et al.*, 2002; Barnett & Bonham, 2004). Cellulose is a linear polymer of anhydro-D-glucopyranose units linked by β-1-4-glycosidic bonds. The covalent bonding within and between the glucose units results in a straight stiff molecule with high tensile strength. In addition, hydrogen bonding occurs between hydroxyl groups of neighbouring

glucose residues of cellulose molecules to form microfibrils (Wangaard, 1981). Frey-Wyssling (1937) who seemed to have coined the term 'Mickofibrillen' first suggested the microfibrillar structure of cellulose (Barnett & Bonham, 2004). Cellulose molecules in each microfibril, are arranged lengthwise with regard to the microfibril axis, but are parallel to each other only in portions. In these portions, that are called crystalline regions or crystallites, the molecules strongly connect to each other by hydrogen bridges. These regions are followed by portions in which cellulose molecules are somewhat disorganized in arrangement-not parallel to one another and not strongly connected. These portions are of low molecular orders, and are called paracrystalline or amorphous regions. These regions are partly or fully occupied by noncellulosic materials like non-pectic polysaccharides, lignin and pectic substances (Donaldson & Singh, 1998; Tsoumis, 1991; Huang *et al*., 2003; Deresse *et al.*, 2003).

→4)-β-Glcp-(1→4)- β-Glcp-(1→4)- β-Glcp-(1→4)- β-Glcp-(1→

Figure. 1.7 *The partial structure of a β-(1→4)-glucan chain seen in cellulose molecule.*

1.7.2 Non-pectic polysaccharides/Glycans

Most glycans are called 'hemicelluloses', a term widely used for all materials extracted from the cell wall with molar concentrations of alkali regardless of structure (Higuchi, 1997; Buchannan *et al*., 2000). Glycans are the noncellulosic pectic polysaccharides that amount to 20-30% of wood (Higuchi, 1997; Kuhad *et al*., 1997; Suurnakki *et al*., 1997). They are smaller molecules than cellulose (Wilson & White, 1981). Glycans in wood are the cementing agents that bind microfibrils together and prevent splits as wood fibres twist, bend and stretch (Mark, 1967). Molecules of non-pectic polysaccharides are parallel to cellulose molecules and connect cellulose and lignin (Tsoumis, 1991). Polysaccharides that cross-link to cellulose microfibrils with hydrogen bonds are called cross-linking glycans (Buchanan *et al*., 2000). They are found mainly in the primary and secondary wall but in small amounts in the middle lamella

(Whistler & Chen, 1991). Glycans are classified into pentoses, hexoses, hexouronic acids and deoxy-hexoses (Fengel & Wegener, 1984; Higuchi, 1997). The pentoses are concentrated in the primary wall and the S_1 and S_3 layers, while the hexosans are more in the S_2 layer (Atalla, 2005). The main chain of non-pectic polysaccharides can comprise of only one unit *homopolymer* e.g. xylan, or have one or two more units *heteropolymer* e.g. glucomannans.

1.7.3 Pectic substances

The pectic substances are the pectic polysaccharides that are also carbohydrates or related compounds. They are prominent in cambial tissues, where they are part of the membrane that separates the young daughter cells produced by the cambium. According to some reports, pectic substances are absent from older wood, however, the prevailing opinion is that they are present, although in small proportions –they are mainly located in the middle lamella and primary wall (Tsoumis, 1991). They could be involved in many functions: determining wall porosity and providing charged surfaces that modulate wall pH and ion balance, regulating cell-cell adhesion at the middle lamella (Buchannan *et al*., 2000). Pectins are a mixture of heterogenous, branched and highly hydrated polysaccharides rich in D-galaturonic acid. Various sugars, such as L-rhamnose, L-arabinose, L-fucose, D-galactose and D-xylose, are linked to the polygalacturonate in pectins (Higuchi, 1997). The polysaccharides that are rich in galacturonic acid can be grouped into two classes: polysaccharide with a backbone of galactopyranosyluronic acid residues such as homogalacturonan and rhamnogalacturonan II (RG-II); polysaccharide with a heteropolymer backbones of galactopyranosyluronic acid residues and rhamnopyranosyl residues such as rhamnogalacturonan I (RG-I). (Carpita & McCann, 2000).

1.7.4 Lignin

Lignin is a major non-carbohydrate component of wood. It is a complex cross-linked three-dimensional polymer formed from phenolic units. The aromatic nature of the phenolic units renders lignin hydrophobic and the three dimensional network provides rigidity to the cell wall and enables it to resist compressive forces (Higuchi, 1997). The

phenylpropanoids, hydroxycinnamoyl alcohols, and 'monolignols' like *p*-coumaryl (4-hydroxy-cinnamyl), coniferyl (3-methoxy-4-hydroxy-cinnamyl), and sinapyl (3, 5-dimethoxy-4-hyroxy-cinnamyl) alcohols (Figure. 1.8) account for most of the lignin network (Whetten *et al.*, 1998; Buchanan *et al*., 2000). The aromatic polymers connect by carbon-carbon and alkyl-aryl ether linkages of guaiacylpropane, syringylpropane and *p*-hydroxyhphenylpropane (Higuchi, 1997; Kuhad *et al*., 1997; Buchanan *et al*., 2000). Since all plants contain *p*- hydroxyphenyl, the plants are classified by their guaiacyl and syringyl content. Gymnosperms mainly contain guaiacyl, whereas woody angiosperms and grasses show a broad range of guaiacyl and syringyl ratios (Adler, 1977; Wardrop, 1981; Higuchi, 1997; Whetten *et al.*, 1998; Buchanan *et al*., 2000). Softwood lignin has monomeric guaiacylpropane units as the major components (>90%), connected by both ether and carbon-carbon linkages (Higuchi, 1997).

Lignin is synthesized from *l*-phenylalanine and cinnamic acids that are derived from carbohydrates through the shikimic and cinnamic acid pathways (Whetten & Sederoff, 1995; Argyropoulos & Menachem, 1997). Lignification proceeds with the conversion of *l*- phenylalanine to form *trans*-cinnamic acid.

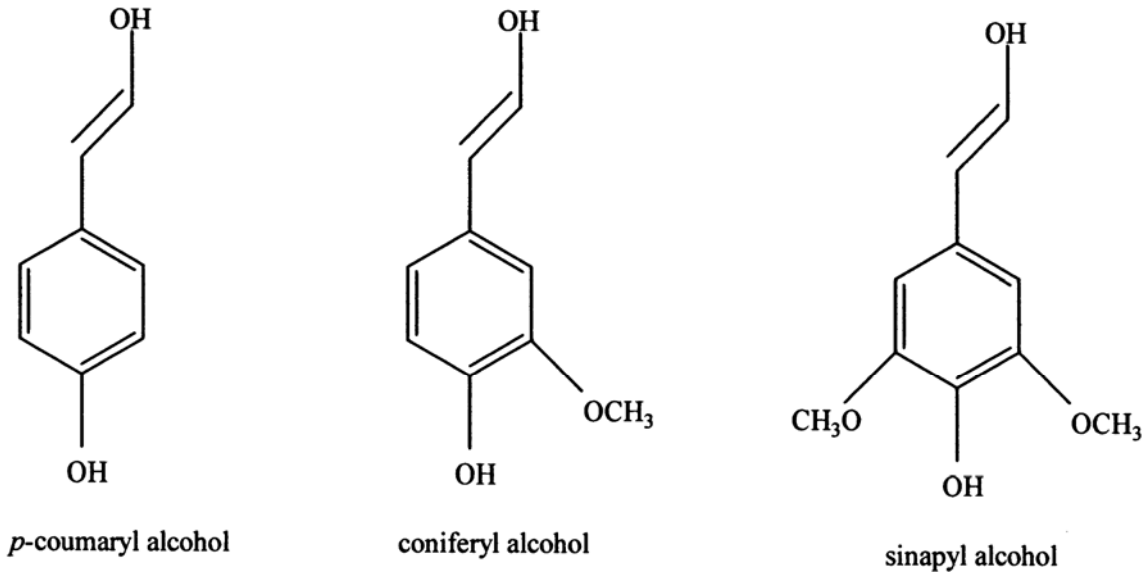


Figure. 1.8 *The structure of monolignols of lignin in wood.*

As lignification proceeds, cinnamic acid is hydroxylated to *p*-coumaric and caffeic acids that are in turn methylated to form ferulic acid by *O*-methly transferase (OMT) (Argyropoulos & Menachem, 1997). Up to this point, the biosynthetic pathways for softwood and hardwoods are believed to be common. However, they seem to diverge beyond this point because OMT enzymes of different functionality and different substrate specificities were found present in softwood and hardwood. One of the reasons that account for the most exclusive presence of guaiacyl lignin in softwoods is that the monofunctional OMT enzyme is inhibited competitively by caffeic acid (Argyropoulos & Menachem, 1997). Another key enzyme responsible for the differentiation of the guaiacyl and syringyl pathways is ferulic acid-5-hydroxylase. The absence of this enzyme in gymnosperms also accounts for the near exclusive formation of guaiacyl precursors in softwoods (Argyropoulos & Menachem, 1997).

Lignin is produced only by living cells. Lignification constitutes the last stage of cell wall development (Jane, 1970; Wardrop, 1981). Completion of lignification practically coincides with consumption of protoplasm and cell death. It is interesting to note that lignin always occurs in association with cellulose, whereas cellulose may be found almost pure in nature (Tsoumis, 1991). Lignification results in filling the pores within the carbohydrate matrix following a sequence from the outer regions of the wall towards the lumen. The amount and chemical characteristics of lignin vary across the cell wall (Donaldson, 2001). In softwood, the lignin content is commonly between 25 to 30% of the dry weight (Jane, 1970; Wilson & White, 1986; Dean *et al*., 1997; Argyropoulos& Menachem, 1997; Lewis *et al*., 1998)). The lignin content in radiata pine is between 24 to 28% of oven dry wood (Uprichard, 1991). In mature wood, lignin is most abundant in the middle lamella, where it may constitute 60 to 90% of the dry weight (Fergus *et al.*, 1969; Jane, 1970; Higuchi, 1997; Argyropoulos & Menachem, 1997) and is least abundant in the secondary wall about 20 to 25% of dry weight. However, 70 to 80% of the total lignin in wood occurs in the secondary wall, as 90% of the wood cell is occupied by secondary wall (Fergus *et al*., 1969; Higuchi, 1997; Argyropoulos & Menachem, 1997; Fromm *et al*., 2003). The lignin concentrations in the cell walls of radiata pine are compound middle lamella 86%, S_2 22% and S_3 53% v/v (Donaldson, 1987). This variable distribution of lignin in the cell walls of pines is a genetically controlled phenomenon

(Donaldson, 1993). Earlywood has a higher proportion of lignin in the cell walls than latewood as the latewood has a thicker S_2 compared to the walls of earlywood (Jane, 1970).

The lignin in the cell wall contributes to compressive strength, water impermeability to the polysaccharide matrix, and cell wall resistance to degradation by pathogens (Whetten & Sederoff, 1995; Lewis *et al*., 1998; Whetten *et al.*, 1998; Gindl & Teischinger, 2002). Several studies have suggested constituents of the backbone of the non-pectic polysaccharides, such as arabinose, galactose and 4-O-methyl-glucuronic acid, are the connecting links to lignin (Adler, 1977; Kuhad *et al.*, 1997). By using transmission electron microscopy (TEM) and scanning electron microscopy (SEM), it was observed that lignin closely followed the cellulose microfibril orientation in the secondary wall (Fromm *et al*., 2003). The observations led to the conclusion that the polymerization of monolignols is affected by the arrangement of the polysaccharides that constitute the cell wall (Fromm *et al*., 2003).

1.7.5 Structural proteins

Structural proteins may also form a network in the wall. There are four major classes of structural proteins, three named for their uniquely enriched amino acid: the-hydroxyproline-rich glycoprotein, the proline rich proteins and glycine rich proteins. The fourth major class of structural protein is the arabinogalactan-proteins (AGPs). AGPs are also called proteoglycans as they can contain more than 95% carbohydrate. All the proteins are developmentally regulated, their relative amounts varying in the tissues and species (Buchannan *et al.*, 2000).

1.7.6 Extractives

In addition to the major cell wall components of cellulose, non-pectic polysaccharides and lignin the wood contains varying amounts of substances termed extractives. Some of the major chemical types are terpenes and related compounds, fatty acids, aromatic compounds, and volatile oils (Wangaard, 1981). Radiata pine wood extractives have low resin content compared to other *Pinus* species, the main resin

components being diterpene, resin acids, fats, fatty acids, sterols and phenols (Lloyd, 1978). The major resin acids in radiata pine are levopimaric, plaustric, pimaric, neoabietic and abietic acid. The predominant fatty acids of glycerides and other esters are oleic and linoleic acid (Uprichard, 1991).

1.7.7 The chemical composition of radiata pine wood

The extractive free wood of radiata pine has a chemical composition of cellulose 40%, lignin 27% and non-pectic polysaccharides 31% (Uprichard, 1991). Studies have shown that the chemical composition is generally similar to other softwoods used commercially (Uprichard, 1991). There is a variation in the chemistry of wood with age. As the tree grows, we see increase in growth, ring numbers and changes in the chemical constituents from the pith to the outer rings (Uprichard, 1991). The estimated data of the range of chemical constituents of inner and outer wood of radiata wood is compiled in Table 1.2.

Components	Composition (%w/w)	
	Innerwood (Rings 1-10)	Outerwood (Ring 16-25)
Lignin	27.6	26.0
Arabinose	1.5	1.0
Xylose	6.7	5.2
Mannose	11.5	12.4
Galactose	4.9	3.1
Glucose	42.4	47.3
Uronic acid, acetyl etc	5.4	5.0
Total	100	100
Lignin	27.6	26.0
Arabinogalactan	5.3	4.0
Cellulose	39.3	43.9
Glucomannan	14.6	15.8
Arabinoglucuronoxylan	8.9	6.9
Acetyl, etc	4.3	3.4
Total	100	100

Table. 1.2 *The variation in the chemical composition of inner and outer wood zones of radiata wood (Uprichard, 1991).*

1.8 The cell wall model

The chemical molecules discussed earlier aggregate to form primary and secondary cell wall layers. However, their organization is not random; there is a pattern in their arrangement that is usually governed by the principles of bonding between the different molecules. The arrangement of molecules involved in primary and secondary wall organization is briefly discussed in the sections 1.8.1 and 1.8.2. The models are general and are not specific to radiata wood alone.

1.8.1 The primary wall model

The mechanical properties of timber depend primarily on the properties of the secondary cell wall. The polymers of the primary wall cell wall account for so small a proportion of wood in terms of weight that they have little influence on properties of wood like strength and stiffness (Newman, 2001). However, the primary wall can affect wood quality by determining shape and size of the cells when they are young. The subsequent deposition of the secondary cell wall uses that shape as a template. Hence, if the primary wall is not of the ideal shape and size, the mature wood will not have ideal properties (Newman, 2001). The primary walls are possibly forming a fundamental framework on which the secondary walls could be laid, and hence, play an important role in the development of secondary wall thickenings. This is because in the differentiating wood cell, the polysaccharides are deposited in successive layers and once the layer has been laid, it remains unchanged (Timell, 1965; Mühlethaler, 1965).

The primary cell wall model as per Dinwoodie (2000) is made of a single layer of randomly arranged cellulose microfibrils. The primary cell wall consists of two or sometimes three, structurally independent but interacting networks. The fundamental framework of cellulose and cross-linking glycans lie embedded in the second network of matrix pectic polysaccharides. The third network consists of structural proteins or a phenylpropanoid network (Buchanan *et al*., 2000).

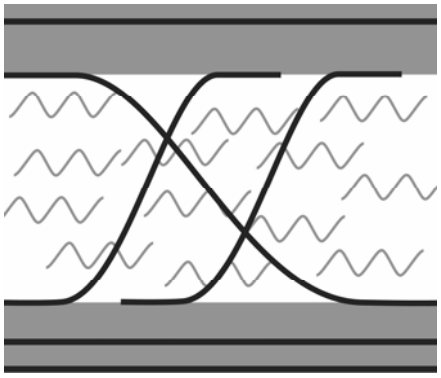


Figure. 1.9 *The primary cell wall model where the xyloglucan occupy only small proportion of the surface area of the cellulose microfibrils (Harris, 2005). Xyloglucans (solid black lines) cross-link adjacent cellulose microfibrils (greyish rectangles), forming a cellulose-xyloglucan network. Pectic polysaccharides (wavy lines) occur between the cellulose microfibrils, forming second network.*

In most of the primary wall models it is assumed, that xyloglucans coat the cellulose microfibrils (Keegstra *et al*., 1973; Ding & Himmel, 2006), while Booten *et al.,* (2004) calculated that only a maximum 8% of cellulose microfibrils surface had xyloglucan adsorbed on to it. However, Harris (2005) is of the opinion that even smaller amounts of xyloglucans adsorb onto surface of cellulose microfibrils (Figure. 1.9). The xyloglucan chain binds to the cellulose microfibrils by hydrogen bonds, and the framework is embedded in a pectin network (Cosgrove, 2001).

1.8.2 The models of lignified secondary walls

The secondary wall consists of cellulose microfibril lamellae that have lignin and non cellulosic polysaccharides attached to them (Figure. 1.10 B; Kerr & Goring, 1975). However, as per Fengel & Wegener (1984), the cellulose core is enclosed by non-pectic polysaccharides (Figure. 1.10 A). Covalent linkages between lignin and non-pectic polysaccharides are very probable, and as non-pectic polysaccharides are likely to be oriented parallel to the cellulose molecules, to which they maybe attached by hydrogen bonds, non-pectic polysaccharides could be considered to act as coupling agents between cellulose and lignin (refer to figure 1.11b). Cross-links similar to the primary walls were

observed in the secondary (S_2) layer of tracheids in *P. thunbergii* (Hafrén *et al.*, 1999), though the morphologies of the secondary wall differed from the primary wall, as the secondary wall has a more compact and ordered cellulose microfibril arrangement (section 1.5). The exact composition of the cross-linking molecules is unknown. However, they could possibly be galactoglucomannans, the main non-pectic polysaccharides in the secondary walls of softwood, or arabino- 4-O-methylglucoronoxylans (Harris, 2005).

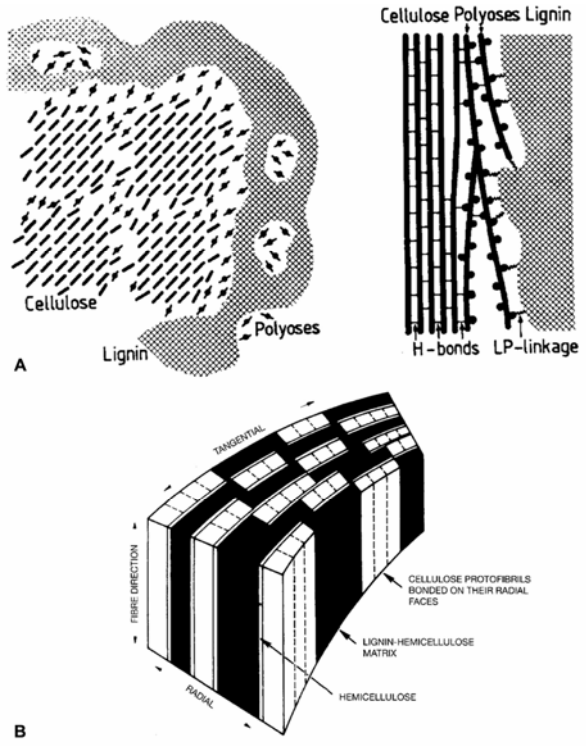


Figure. 1.10 *A) The arrangement of cellulose, non-pectic polysaccharides and lignin in the wood cell wall. As per this model, the cellulose core is enclosed by non-pectic polysaccharides that could act as a coupling agent between cellulose and lignin (Fengel & Wegener, 1984). B) According to this model the cell wall consists of blocks of cellulose microfibrils to which blocks of lignin and non-pectic polysaccharides are attached tangentially (Kerr & Goring, 1975).*

All these non-pectic polysaccharides can hydrogen bond to cellulose microfibrils, and those not associated with cellulose as part of the first network form part of the second network equivalent to the pectic network of primary walls. The second network is probably composed of glucuronoarabinoxylans that have backbones with higher degrees of substitution than do those associated with cellulose microfibrils. The glucuronoarabinoxylans are probably cross- linked by ferulic dimers (Harris *et al*., 2005). During lignification, the monolignols polymerise to form lignin. The ferulic acid ester linked to non-pectic polysaccharides in primary walls of gymnosperms may function to nucleate lignification (Carnachan & Harris, 2000; Harris, 2005). The lignin is covalently linked to cellulose and xylans in ways that indicate that the orientation of polysaccharides may serve as a template for lignin patterning (Buchanan *et al*., 2000). Further links proposed between the non-pectic polysaccharides and lignin are direct ester links involving uronic acid residues and direct ether links (Iiyama *et al*., 1994).

1.9 Different approaches used to study xylem development

The organization and complexity of the vascular tissue make the study of tracheid differentiation a rather challenging one. Xylogenesis presents an ideal system to study the fundamental aspects of plant cell differentiation and pattern formation (Chaffey, 2002b; Ye, 2002). The process of tracheid development provides a simple analytical model system for plant cell differentiation as it undergoes distinctive cytological changes; there is patterned secondary wall formation and loss of protoplast (Kuriyama & Fukuda, 2001). Most of the studies conducted so far have been in more amenable systems than trees (Chaffey, 2002b). Tracheid differentiation has been investigated in intact plants, cell suspension cultures and *in vitro* systems using explants or callus (Brown, 1964; Hertzberg *et al*., 2001; Chaffey, 2002b; Ye, 2002).

1.9.1 Model systems used for study of xylogenesis

It is impossible to study every organism in detail and understand its biology and hence, a handful of *model species* have been selected for detailed study in the hope that they are representative of wider range of life forms (Chaffey, 2002a; Przemyslaw, 2004).

1.9.1.1 *Arabidopsis thaliana*

This is one of the most popular model systems for plant research and carries a heavy responsibility as a model species (Chaffey, 2002b; Ye, 2002). It is a small plant with a life cycle of 6-8 weeks, self-pollinated, and easy to transform, hence, making it an excellent system to use in controlled studies (Hertzberg *et al*., 2001). Large collections of its mutants have opened a completely new avenue for understanding the molecular mechanism for vascular development and biosynthesis of cell wall material (Reiter, 1997; Hertzberg *et al*., 2001; Ye, 2002). *Arabidopsis* develops vascular cambium and wood, however, lacks the features of a tree (Chaffey, 2002a). It has its limitations as it lacks the trees secondary vascular system, such as seasonal variation of earlywood and latewood production, the seasonal cycle of cambial dormancy-activity and the production of heartwood (Chaffey, 2002b).

1.9.1.2 Poplar

Poplar has recently been promoted as the model for angiosperm (hardwood) trees (Mellerowicz *et al*., 2001; Hertzberg *et al*., 2001; Chaffey, 2002a; 2002b; Ye, 2002). It is a fast growing, small genome, easily transformable tree that enables genetic approaches to be used successfully (Hertzberg, 2001). Though *Poplar* has helped in understanding the process of xylogenesis, it does not encompass the full range of tree types like gymnosperms and tropical ring porous forms of wood (Chaffey, 2002b).

1.9.1.3 Coleus

The stem from coleus was used to study the role of auxin and cytokinnin in induction of vascular development (Ye, 2002). The advantage of the system is that the stems are big enough for easy excision and subsequent analysis of effects of external factors on vascular differentiation. However, the system has been used mostly for physiological studies (Ye, 2002).

1.9.2 Tissue culture

Tissue culture techniques have become a valuable tool in the study of growth and differentiation in plants (Brown, 1964). Tissue cultures of woody plants are usually

established by aseptically transferring small fragments of tissue from the pith, phloem or cambial zone to a synthetic medium containing a carbon source, essential inorganic nutrients and other growth factors. Generally, when the parenchymatous cells of the excised tissue are placed in culture they undergo rapid proliferation resulting in the formation of callus. Pieces of this initial callus may then be successively sub- cultured to study specific phases of growth and development (Brown, 1964; Fukuda, 1996; Möller, 2003).

1.9.2.1 Suspension cultures

One of the most successful suspension cultures developed with respect to xylogenesis is the *Zinnia* system. It has been 20 years since the *Zinnia* culture system was used to study tracheary element formation (Pesquest *et al*, 2003). The *Zinnia* system is based on cultured *Zinnia* leaf mesophyll cells that can be induced to transdifferentiate into tracheary elements in the presence of auxin and cytokinnin (Domingo *et al*., 1998; Hertzberg *et al*., 2001; Ye, 2002). The early process of transdifferentiation involves the dedifferentiation of cells and subsequent differentiation of dedifferentiated cells into tracheid element precursor cells that lead to development of tracheid development (Fukuda, 1996). The advantage of the system is that it is homogenous, with a possibility of synchronising the entire re-differentiation process. This can be of help when it comes to sampling at specific stages of tracheid differentiation and the possibility opens of adding or withdrawing substances at any time during the re-differentiation process (Hertzberg *et al*., 2001; Milioni, 2002; Ye, 2002). A number of genes have been isolated and characterised using this system (Ye, 2002).

Isolated protoplasts from the cambial zone and developing xylem of *Pinus* species have also been used for furthering the understanding on tracheid differentiation (Leinhos& Savidge, 1993).

1.9.2.2 Callus cultures

In callus cultures obtained from the cambia of woody plants, cultures grown on standard nutrient media show various stages of differentiation ranging from undifferentiated callus to cells with apparently normal shoots and root (Brown, 1964).

Various types of tissue explants and different species may develop differently on nutrient media of essentially the same consistency (Brown, 1964). The longitudinal bark strips of *Populus trichocarpa* and *Pinus strobus* separated from bole wood have been successfully cultured to study the process of differentiation (Brown & Sax, 1962); likewise, tracheids have redifferentiated from parenchyma cells in the hypocotyls of *Pinus pinea* (Kalev & Aloni, 1998). *Pinus radiata* callus cultures have been derived from hypocotyl segments and xylem strips and used to study tracheid differentiation (Moeller, 2003).

1.9.2.3 Stem segments

Stem segments have been used as an experimental system for studies on vascular differentiation. Radiolabelled or other precursors in the aqueous solution can be infiltrated into the segment bases and, following an incubation period, the tissue from the plants stem segments can be used for various analyses and studies (Savidge, 2000). Stem segments were also found useful in some morphogenetic studies that require longer experimental periods. Certain studies on cambial activity were carried out by dipping the ends of the stems in various solutions or applying substances to their cut surfaces in lanolin paste (Brown & Wodzicki, 1969).

1.9.2.4 Organ cultures

Organ cultures are stem explants grown on defined media. This system has been used successfully to study the effect of phytohormones on xylogenesis, as well as study the formation of bordered pits (Savidge, 1993; Leitch & Savidge, 1995). Savidge (1983) grew stem 'chips' of *Pinus contorta* on basal media, and within the chips, cambial cell division and tracheid differentiation occurred. Leitch (1999) used the same method to study tracheid development in *E. globules.*

1.9.3 Computational models or 'virtual plants'

Computational plant models are the latest trendy tools in the world of biology. They are seen as useful tools for understanding complex relationships between gene function, plant physiology, plant development and resulting plant form (Przemyslaw, 2004). Advances in the technology have led to sophistication in software, so that now it is

possible to simulate highly complex processes seen in plants. The simulation software may be general purpose, intended to capture a variety of developmental processes depending on the input files, or special purpose, intended to capture a specific phenomenon. The input data range from a few parameters in models capturing fundamental mechanism to thousands of measurements calibrated to descriptive models of specific plants (Przemyslaw, 2004).

There are differences of opinion on the value of virtual plants. The differences exist on fundamental issues like role of theory in biological understanding, the usefulness of applying chains of mathematical deductions to biological data, and the appropriateness of transplanting research methodologies from physics to biology (Keller, 2002).

1.10 Assessment of organ culture as a tool to study xylogenesis

The organ culture method offers hope for progress of tracheid differentiation study *in vitro*. In the studies conducted using this method it was observed that cambial cells underwent periclinal divisions to produce radial files of secondary walled, lignified, bordered pitted and earlywood tracheids just like in intact trees (Savidge, 1983; 1993). However, this method has its limitations like other tissue culture methods. The physical environment experienced by the cells in the organ culture may be abnormal in relation to the cells *in situ*. The cells in organ culture do not experience negative pressure potential or strong osmotic forces in the phloem. The axial translocation through phloem and xylem or radial translocation through rays, as normally occurs *in arbor* probably does not occur here (Leitch & Savidge, 2000).

Organ culture is a system that mimics the process of xylogenesis in an intact tree (Leitch & Savidge, 2000), unlike other tissue culture methods, where there is development of tracheid like cells. The other advantage of organ culture is ease of monitoring and control of environmental and nutrient conditions (Leitch & Savidge, 2000). Xylogenesis is able to proceed in organ culture without interferences from other processes such as phytohormones (stress response) produced in another part of tree in response to changes in the experimental setup. The more control that can be exercised

over the experimental conditions the more reliable the data obtained, leading to better understanding of xylogenesis (Leitch & Savidge, 2000). The changes taking place can be monitored along a single cell file as, different stages of xylogenesis are displayed along a single cell file giving a sort of continuum view of xylogenesis as it takes place in the culture. Hence, organ culture was the tool of choice to study xylogenesis in radiata pine in this project.

1.11 The role of auxin in xylogenesis

It is an established fact that auxin plays a critical role in the regulation of plant growth and development. Auxin has been implicated in a wide variety of developmental processes in plants including elongation, growth and differentiation of vascular tissue (MacDonald, 1997; Uggla *et al.*, 1998; Tuominen *et al.*, 1997). The vascular cambium is responsible for xylem formation, which constitutes the bulk of secondary growth (Tuominen *et al*., 1997). The role of auxin has been investigated in many studies and they show that it affects cambial growth, xylem, phloem production and size, and secondary wall thickness of the xylem elements (Roberts *et al*., 1988; Savidge, 1996; Little & Wareing, 1981; Sundberg & Little, 1990; Sundberg *et al*., 1994; Uggla *et al*., 1998). These will affect the secondary growth that will finally determine the critical wood properties like annual ring width, earlywood and latewood formation (Wilson & Howard, 1968; Gregory, 1971; Wodzicki & Zajaczkowski, 1974; Denne & Wilson, 1977; Dodd & Fox, 1990; Ridoutt & Sands, 1994). The physiological relevance of auxin in regulating cambial growth was demonstrated after experiments with exogenous auxin and auxin inhibitors in shoots of *Pinus sylsvestris* (Sundberg & Little, 1990; Sundberg *et al.*, 1994). There is a strong correlation between auxin concentration and cambial growth (Uggla *et al.,* 1998). From these studies, it is evident that auxin has a role, not only in the rate of physiological processes but also as a morphogen conveying positional information during xylem development.

1.12 The role of boron in xylogenesis

Lewis (1980) proposed that boron was a prerequisite for the evolution of vascular plants from pre-vascular plants. Boron is an essential micronutrient for higher plants. Boron seems to be affecting plants in various ways, such as growth, differentiation, membrane permeability, and influencing enzymes involved in the metabolism of products like polyphenols, lignin, carbohydrates, auxin and nucleic acids (Neales, 1964; Coke & Whittington, 1968; Shkolnik, 1984; Cohen & Albert, 1974; Birnbaum *et al*., 1977; McIlrath & Palser, 1956; Lee & Aronoff, 1967; Rajaratnam *et al.*, 1971). Deprivation of boron in plants leads to changes like growth inhibition, death of the apical meristem and abnormal or lack of vascular differentiation (Goldbach *et al.*, 2001).

Recent evidence suggests that boron plays an important role in primary cell walls (Brown & Hu, 1997, Matoh *et al*., 1998). Boron in primary cell walls cross-links the pectic polysaccharide rhamnogalacturonan-II (Kobayashi *et al.*, 1996; O'Neill *et al.*, 1996). Rhamnogalacturonan-II (RG II) exists in all higher plant primary walls predominantly as a dimer that is covalently cross-linked by borate to create di-ester (RG-II-B-RG-II) (Ishii & Ono, 1999). Dimer formation results in a cross-linked pectic network because RG-II is covalently inserted within homogalacturonan chains (O'Neill *et al.*, 2001). Studies have demonstrated that the borate localized in cell walls, cross-links two RG-II to form a dimer (RG-II-B-RG-II), these have been found in radish root (Kobayashi *et al*., 1996), sugar beet (Ishii & Matsunaga, 1996), and bamboo shoot (Kaneko *et al.*, 1997). RG- II from gymnosperms was isolated and characterized from cell walls of suspension cultured Douglas fir (Thomas *et al*., 1987). Shimokawa *et al*., (1999) also isolated RG-II-B complex from etiolated whole hypocotyls of *Pinus densiflora* and borate-linked glycosyl residues in RG-II-B. The results support the universality of borate location in the cell wall among dicots, graminaceous monocots, and coniferous gymnosperms.

The localization of boron in the cell wall and its association with cell wall pectins and the effect of boron on cell wall extensibility suggest that boron plays a critical role in cell wall structure of plants. Numerous investigations have noted changes in cell wall structure as a result of boron deficiency especially thickening of the cell wall coupled with increasing disorganization of middle lamella. This led to the suggestion that the

physiological importance of boron is to cross-link cell wall pectins (Ishii & Matsunaga, 1996; Kobayahsi *et al*., 1996; O'Neill *et al*., 1996). Such a cross-linked pectic network is likely to have a role in regulating the mechanical and biochemical properties of the wall (Fleischer *et al*., 1999; Ishii & Matsunaga, 2001)

Boron deficiency results in changes in cell wall structure for example swelling of the cell wall and formation of small irregularly shaped cells (Brown & Hu, 1997; Matoh, 1997). Boron deficient cells have increased pore size indicative of a disorganized pectic network and may result from the absence of borate ester cross-linked RG-II (Fleischer *et al.*, 1999). Ishii *et al.*, (2001) in their experiment were able to show that walls of boron deficient cells were swollen. On adding boric acid to the boron deficient plants, there was a decrease in the wall thickness. They concluded that on adding boric acid there was rapid formation of borate ester cross-linked RG II dimmer (RG-II-B-RG-II) from m RG-II and repacking of the matrix into thin cell wall.

If boron was a necessary component for membrane integrity, deficiency would lead to inhibition of growth. Since the membrane is now believed to play more than passive role in the growth and development of cell, any impairment of its structure could result in abnormal growth. The boron effect could be due to its interaction with some membrane components that are needed to maintain structural integrity (Tanada, 1974). This is where we see a connection between boron and auxin.

1.13 The relationship between auxin and boron

Some of the symptoms of plants that are deficient in boron are sufficiently similar to those deficient in auxin. Boron deficiency symptoms are shoot and tip dieback. Shoot dieback is a condition where the main shoot or the leader dies and tip dieback is the loss of the apical bud (MacLaren, 1993).

According to the some researchers (Timell, 1986) boric acid has a growth regulation effect if not directly on IAA, it might exert an influence via an enzyme system, probably peroxidase, which has been known to operate in the destruction of IAA in the tissue. Conversely, some researchers believe that an increase in levels of endogenous auxin is associated with boron deficiency in a number of species (Coke & Whittington, 1968). Auxin at high concentrations inhibits ion transport, and uptake and absorption of

B^+(Pollard *et al*., 1977). From the evidence in the reports, one can see that boron and auxin intermesh with the each other and play an important role in growth and development of woody tissue.

1.14 Intra-ring checking, a wood defect of *Pinus radiata*

The term defect when applied to wood refers to any irregularity or deviation from the qualities that make wood unsuitable for a particular purpose (Panshin & de Zeeuw, 1970). Intra-ring checking is a wood defect that usually makes its appearance in kiln dried wood (Panshin & de Zeeuw, 1970), though rarely seen in freshly felled trees (Kimberley& Conchie, 2004). Checking is a seasoning or drying defect, as it occurs in the wood mainly during drying (Panshin & de Zeeuw, 1970).

The intra-ring check extends radially through the earlywood region of the growth ring (Booker *et al.*, 2000), and rarely extends across the latewood zone (Cown *et al*., 2003) (Figure. 1.13). The check occurs at right angles to the growth ring orientation (radially) (Cown *et al*., 2003). The main factors identified in the literature that could contribute to checking include intrinsic properties, genotypes of the trees, site, environmental conditions, and the processing methods (Cown *et al*., 2003).

Some of the characteristics of the checked wood were lower density (the wood measured was from breast height outerwood cores), wide annual rings, thin cell walls, delayed latewood formation and delayed lignification (Ball *et al*., 2001). There is a close relation between checking and cell wall thickness of S_1, S_2 and S_3, microfibril angle, tensile strength of compound middle lamella and degree of water stress that cell wall layers undergo (Booker, 1993; Booker & Sell, 1998). The external conditions conducive for the formation of check prone wood could possibly occur when the environmental conditions are conducive to producing wide earlywood of low density and a high proportion of sapwood (Cown *et al*., 2003).

products (Cown *et al*., 2003). Hence, there is a need to understand this wood flaw, the reason for it to be one of the important objectives of this project.

1.15 Brief discussion of the project goals

Wood quality can be defined as properties of wood that are of importance to the industry (Label *et al*., 2000). Wood quality until now was usually considered as large, straight stems free of defects. These days, however, genetic and silvicultural trials are being evaluated in terms of fundamental wood and fibre properties (Downes *et al*., 2000). A wood defect like checking is certainly undesirable wood trait. In order to eliminate it there is a need to understand it, and that is one of the aims of the project.

One major difficulty of research in the field of wood quality is the considerable differences in time-scales between industrial needs and tree development (Label *et al*., 2000). Organ culture seemed an ideal method for the project as it is an *in vitro* method that mimics development of wood in the intact tree, and furthermore the experiments could be carried out in shorter duration of time and in a more efficient and controlled manner.

The boron content of New Zealand soil is generally at low level. Boron deficiency exists in a wide range of soils on eastern side of both islands (McLaren, 1993). This makes it all the more important for us to understand the role of boron in radiata pine plantations. Radiata pine shows rapid growth and hence the assumption that it could have high endogenous auxin levels that might influence and even aggravate the symptoms of boron deficiency (refer to section 1.13). The hormone auxin plays an important role in cambial activity. Hence, experiments in the project were conducted with the aim to understand the affect of auxin and boron on radiata pine cambial activity and xylogenesis using organ cultures that will be discussed in detail in chapter 6 and 7.

Radial growth is directly related to cambial activity, while the wood mechanical properties are related to wood anatomy and xylem structure (Label *et al*., 2001). There is a likelihood that factors like auxin and boron influence wood formation and that in turn will affect the wood quality. Hence, the project was designed to understand the intra-ring checking in wood, and gather more data on the impact of auxin and boron on xylogenesis

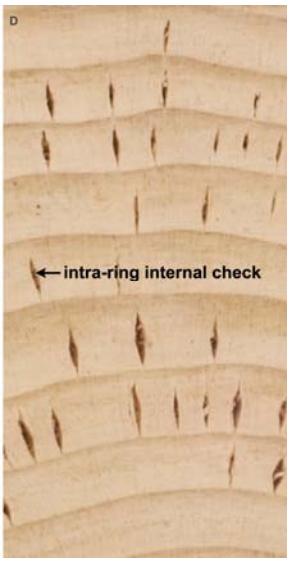


Figure. 1.11 *Digital image of a partial oven-dried disc of radiata pine. The intra-ring check is seen to span the earlywood and end in or at latewood boundary on either side.*

The site factors that seem to lead to check prone wood are drought years, and poor microsite drainage (Ball *et al*., 2001). Similarly Olykan *et al*., (2003) found in their study that tree growth and soil nutrient supply like boron, could play a significant role in intra-ring checking. Kumar *et al*., (2004) conducted a study to see the influence of genotype on wood formation when grown at different sites. They found that there was a large tree-to-tree variation at each site. The proportion of trees free from checking also varied at different sites. This could mean that a range of sites could be identified for planting for future resources for appearance grade timber. They also found that the checking of wood was under moderate genetic control. Thus, it is possible to do selective breeding for reducing checking in radiata wood pine (Kumar *et al*., 2004).

The basic predisposing factors clearly play a role in checking but processing and product variables could play a role in check development in wood. During processing, it was found that factors that exert their influence on checking could be board dimensions and drying temperature (Cown *et al*., 2003).

Intra-ring checking in radiata pine has become a relatively common phenomenon and it could have a dramatic influence on the perceived value of crops and lumber

in radiata pine wood, hoping that the research will not only add knowledge but also help in finding ways of improving wood quality of radiata pine.

1.16 Format of this thesis

Chapter two will present the result of the study that was undertaken to determine the location of check. **Chapter three** describes the differences of the tracheid dimension of the checked and the non-checked wood. **Chapter four** highlights results of the comparative study that was conducted to see if the checked wood differed from non-checked wood with respect to lignin and pectin levels in the cell wall layers. **Chapter five** examines the influence of rays, resin canals and pits on checking. **Chapter six** describes the development of method for organ cultures of radiata pine and the role of auxin in xylogenesis of radiata pine. **Chapter seven** describes the organ culture study that conducted to understand the role of both auxin and boron on xylogenesis in radiata pine. **Chapter eight** presents an overall summary of studies undertaken in this project to understand intra-ring checking and efficacy of the organ culture method developed.

Chapter Two

The point of failure in checked wood

2.1 Introduction

Intra-ring checking is a wood quality defect that appears during drying. The flaw is not detected until the last stage of processing and results in down grading top quality appearance products to firewood (Miller, 2004). Checking started becoming commercially important with increased usage of younger trees and recycling of wood products (Simpson *et al.*, 2002). There is no explanation as to the cause of checking (Miller, 2004). There are a number of factors that have been put forth as the possible reasons for the development of checks in wood such as wood characteristics (like wood density), genetic stock, climatic and soil conditions, silviculture, and processing parameters (also refer to section 1.14). Chapters two, three and four of this thesis are dedicated to the study of wood characteristics of checked wood, aimed at determining the attributes of the wood that could make it more susceptible to checking. The understanding of the wood characteristics associated with checking would help us gain insight to the problem and may help in solving this wood quality issue.

This chapter discusses the study that was conducted to try to determine the site of checking in radiata wood. Intra-ring checking usually occurs in the earlywood region and ends in or at the latewood region of the growth rings (refer to section 1.14). Hence, the observations for this study were mostly carried out in the earlywood cells of the checked wood to locate the point of failure in wood.

2.2 Material and Methods

2.2.1 Experimental Design

Thirteen half discs cut from 60 mm oven-dried discs of radiata pine were kindly provided by Wood Quality Initiative Limited, New Zealand (WQI Ltd) to carry out the studies on intra-ring checking in radiata pine. Nine of these discs displayed varying intensities of checking, and four discs did not show any signs of checks in any of the growth rings (Figure. 2.1). The discs without any check were categorised as non- checked discs (Figure. 2.1C), while the discs with checks were categorized into severe (Figure 2.1A) and moderate (Figure 2.1B) checked discs depending on the number of checks that were observed in the growth rings and the number of growth rings affected. The observations for this study were restricted to growth ring seven to minimize the variations that occur across, and between growth rings.

2.2.2 Sample preparation for scanning electron microscopy (SEM)

From each checked disc, five checks were collected from growth ring seven using a hammer and chisel. If five checks were not available, checks were collected from an adjacent growth ring; usually ring eight was used. These checks were then observed along the transverse and radial surface. The checks for the radial surface were carefully split so as not to disturb the tear in the cell walls. The transverse parts of the checks for the investigation were selected and the surfaces trimmed with sharp razor blade.

Out of the five samples, collected two samples from each disc were subjected to lignin extraction. Two checks from each of the eleven discs were subjected to methylamine extractions. The checks were left in approximately 2 mL (enough to immerse the check) of methylamine (40% MERCK- Schuchardt, German) for 48 hours to extract lignin in fumehood. This procedure was carried out to make cellulose microfibrils easier to visualize (Chaffey, 2002a). On removal from methylamine the samples were rinsed twice in water and then put through ethanol series (10, 20, 30, 50, 70, 80, 90 (v/v) and 100% ethanol) followed by an isoamyl acetate (Analar, BDH, England) series (10, 20, 40, 60, 80, 90, (v/v) 100% isoamyl acetate). The samples from the 100% isoamyl

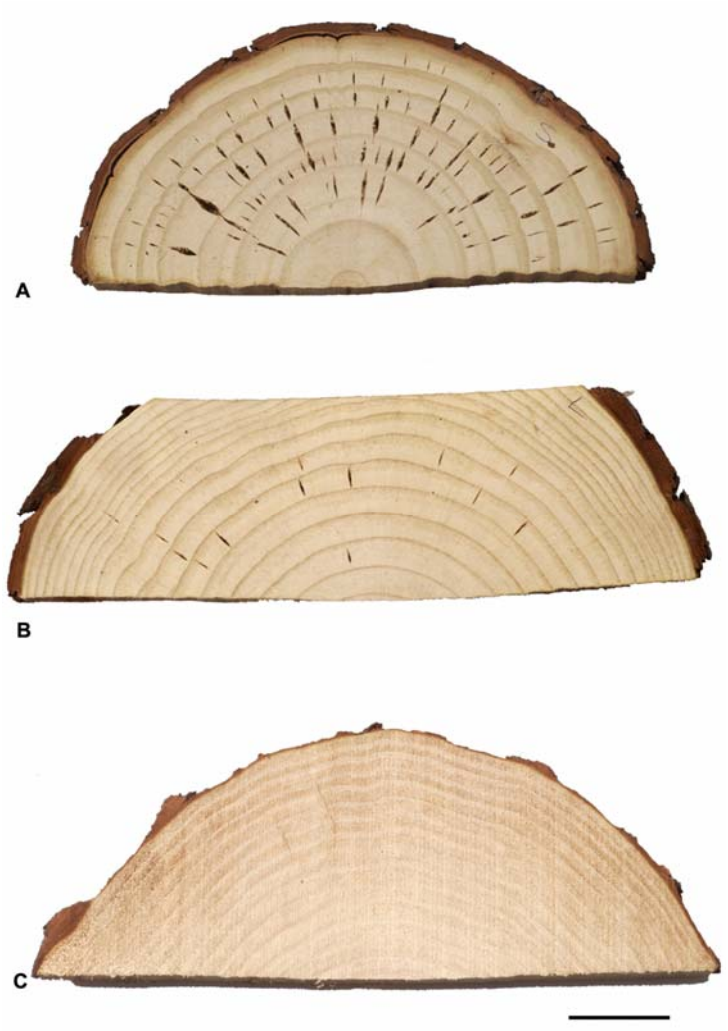


Figure. 2.1 *Digital pictures of the oven dried discs of radiata pine wood. The discs were categorized into severe and moderate checked discs depending on the number of checks seen and the number of growth rings affected. In severe checked (A) disc more number of checks in a single growth ring (a false growth ring can also be seen in this sample) and more growth rings were affected compared to the moderate disc (B). In the non-checked discs we see no checks in the growth rings were seen (C). Scale bar=5cm.*

acetate were subjected to critical point drying (critical point drier model CPD 750, Emscope Laboratories Ltd., England), and mounted on scanning microscopy stubs and sputter coated with gold palladium for two minutes at 20 miliampheres to give a coating of approximately 100 °A thick.

They were observed with a Leica S440 scanning electron microscope, at magnifications ranging from 65 to 15,000x. On observing the electron micrographs there was not much of a difference between the samples that had lignin removed and those that still had lignin.

2.2.3 Image analysis

The electron micrographs were marked for the various cell wall layers exposed along the radial surface in Adobe Photoshop (version 6). The surface area occupied by each wall layer was then determined using Image Pro Plus (version 4.5 for Windows, Media Cybernetics Inc, USA).

2.3 Results and Discussion

The intra-ring checks seen along the transverse and radial planes of the severe and moderate checked radiata pine wood were studied to locate the point of the cell wall failure. These observations could help us to gain more insight into the cause of check development.

2.3.1 Determining the point of failure in the cells of checked wood cell

The cell wall layers along the checked surface were identified using the relative position of the exposed cell wall layers and their characteristic microfibrils arrangement.

Towards the cell lumen, the order of arrangement of the cell wall layers in the tracheids with respect to one another is the cml followed by the S_1, S_2 and the S_3 secondary cell wall layers. This stacked pattern of the wall layers can be seen in Figure. 1.4. Each of the cell wall layers have their characteristic cellulose microfibril arrangements (refer to section 1.6.3), that served as a valuable guide to identify the cell wall layers exposed along the point of failure.

2.3.2 The crack occurred at the cml/S_1 interface.

In order to understand and be able to describe the failure phenomenon in checking the terminology used by Côté and Hanna (1983) was adopted. When a failure occurs in wood cells three types of breaks are recognized: intercell, intrawall and transwall. Intercell failure occurs at the middle lamella, and is simply separation of cells at this junction. Intrawall failure refers to failure within the secondary wall and in most instances, it is at the S_1/S_2 interface or close to it. When the rupture of the wall is complete (when the fracture path cuts across the wall) the failure is described as transwall.

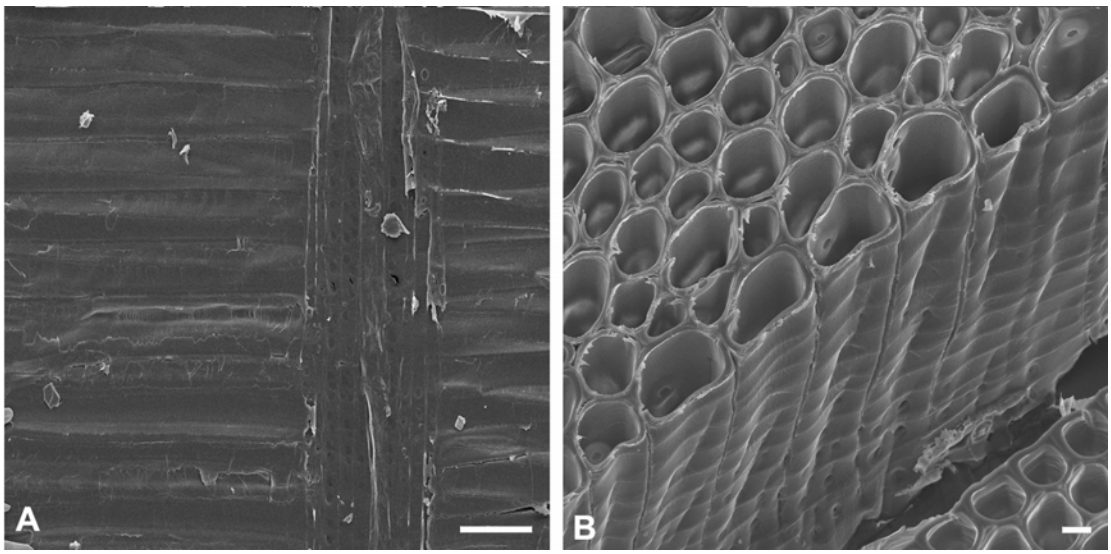


Figure. 2.2 *SEM images of the radial surface (A) and transverse surface (B) of the intra-ring checks of the radiata pine wood. The exposed checked surface is mostly smooth; hence, the intra-ring check is usually an intercell failure at the cml between two adjacent cells. A) Scale bar=100μm and B =20μ.*

SEM images showed that the intra-ring checking involved the failure of cell wall layers between adjacent cells. Hence, it seems that the check is most likely an intercell failure. Few failures in the cell wall layers went across a cell wall. Thus, it is highly likely that intrawall and transwall failures do not play a role in checking. The checks nearly

always had a smooth surface (Figure. 2.2), which indicate that the split developed at the cml. According to Donaldson (1995), cell walls may undergo failure within cell wall layers and such fractures usually tend to follow the lamellate structure of cell wall matrix, and often produce a smooth surface. The image analysis of the exposed radial surface cell wall layers showed the exposed surface was occupied by the cml the most followed by S_1 and rarely the S_2 (Figure. 2.3). Hence, most of the fractures probably occurred at the boundary of cml/S_1 cell wall layers. These observations find further support from the fact that in the transverse surface of the checks the secondary cell wall layers were seen to be usually intact (Figure. 2.2B). Similar observations were reported in other studies where the intercell failure of wood mostly takes place at the cml and S_1 layers (Cousins, 1974; Cote & Hanna, 1983; Donaldson, 1995; 1996; 1997; Singh & Donaldson, 2000; Cown *et al.,* 2003).

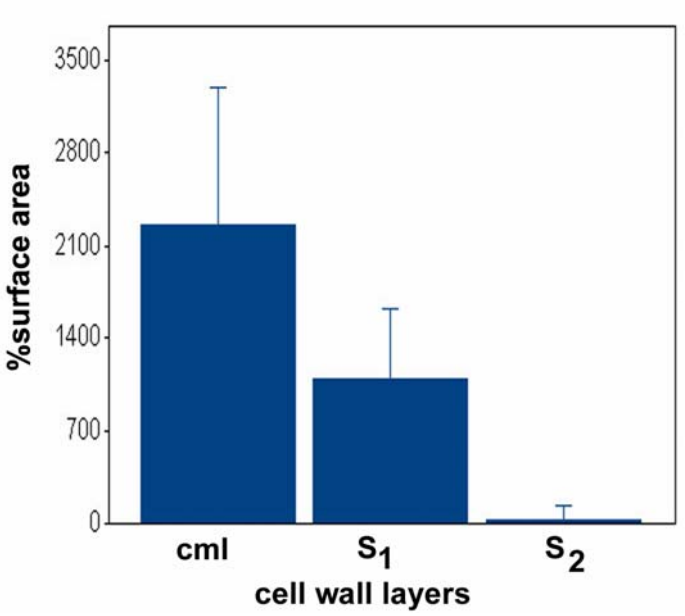


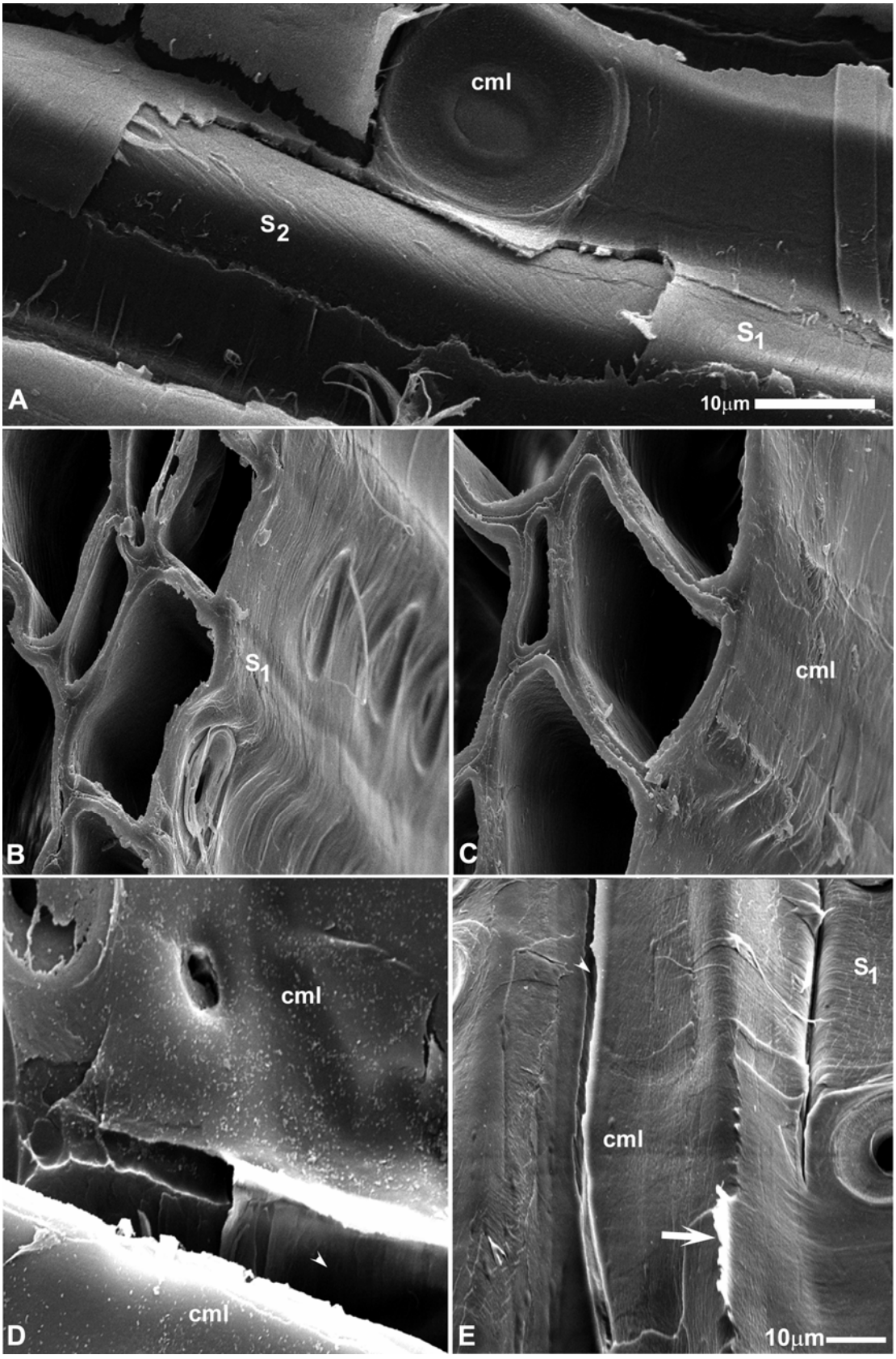
Figure. 2.3 *The proportion of the radial face of the check occupied by cml, S_1 and S_2 wall layers. The bars indicate the standard deviations.*

The S_2 layer was exposed only in one check and the S_3 layer was not displayed in any of the exposed surfaces observed. Only in one check intrawall failure between S_1 and S_2 layer was observed (Figure. 2.4A). Along some of the exposed checked surfaces there were some fractures detected within the S_1 layer. When this occurred, a small piece of S_1 layer was seen to peel off from the exposed surface giving the checked surface a rough appearance (figure 2.4E arrow). S_1 and S_3 layers were described elsewhere, as protruding from the fracture surface (Donaldson, 1995).

The cell walls split from each other at the interface due to differences in the nature of cell wall layers. This effect could be due to the change in microfibril orientation in the cell wall layers (Donaldson, 1997). The cml had fewer cellulose microfibrils arranged randomly (Figure. 1.5), while the S_1 wall layer that lies adjacent to it has compactly arranged microfibrils (see section 1.6.3). Hence, it seems that the failures take place within the matrix and avoid splitting across the cellulose microfibrils wherever possible (Donaldson, 1997). Donaldson, (1996) estimates that the fracture within the matrix requires only 15% of the energy required to fracture across the microfibrils. Fracture across the microfibril axis involves breaking of chemical bonds within the individual cellulose molecules (Donaldson, 1997). Hence, it is plausible that the failure occurs and follows the path of least resistance involving the cml that is more matrix rich. Cousin (1974) pointed out that splitting in the cell wall occurs at the edge of cml near its junction with the secondary (S_1) wall and takes place when the stress in the cml exceeds the strength of the amorphous matrix of which it is composed. He also stated that the fracture occurred in the hemicellulose rich primary cell wall layer or S_1 layer rather than the lignin rich middle lamella, which suggests that the intermolecular bonding of lignin might be stronger than that of non-pectic polysaccharides and that the fracture occurs in a predominantly hydrogen bonded region of cell wall. Therefore, it seems likely that the precise location of the check in the wood cell could be at the primary cell wall and S_1 cell wall interface.

The cell wall and its chemical composition have also been shown to be implicated in fracture development and formation of checks in wood (Donaldson, 1995; Singh & Donaldson, 2000).

Figure. 2.4 *The scanning electron micrographs of the radial face (A, D, E) or the transverse face (B, C) of the check were analysed to determine the location of check. The location of the failure in the cell wall was determined from the wall layers exposed at the checked surface. cml, S_1 and S_2 layers were seen along the exposed checked surface as indicated on the micrographs. All three wall layers were present in A. In C and D, the fracture occurred between tracheids at the cml. cml was the predominant wall layer detected on the exposed checked surface. The S_1 wall layer with its characteristic horizontal cellulose microfibril pattern was seen in B and E. Tufts of wall (large arrow) were seen to stick out of the checked surface and the spaces created when such tufts of wall were removed were also seen (small arrow).*


cml
S2
S1
A
10μm
S1
B
cml
C
cml
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D
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cml
E
10μm

According to Donaldson (1995), reduced lignin content in the cml could contribute to differences in the fracturing behaviour in wood, especially sudden changes in lignin concentration across cell wall layers, such as cml/S_1 or S_1/S_2 boundaries.
Since the cml has a higher lignin concentration than S_1 (refer to section 1.7.4), this region tends to fail more easily during conditions of stress. If lignin content is reduced in cml region, then the differential lignin concentration between the cml/S_1 will also be reduced, and then there is possibility of cell wall fracture favouring other cell wall sites (Donaldson, 1995).

When the cml was chemically delignified then it was found that fracture formation took place in the cml (Lai & Iwamida, 1993).The frequency of fracturing also depends on the relative tensile strengths of the cml/S_1 or S_1/S_2 interfaces, and a decrease in the lignification could account for decrease in their tensile strength (Donaldson, 1997). Lignin is known to contribute to the compression strength of wood including the ability of the cell walls to resist collapse due to negative pressures that develop within cells during transpiration and in timber during drying (Singh & Donaldson, 2000). If there is a decrease in the lignin content particularly in the cell wall boundaries then there will be an increase in the tendency for cellular collapse making the wood more susceptible to temperature induced collapse (Singh & Donaldson, 2000) and this might lead to formation of checks in wood.

From the discussion above it seems that cell walls split from each other at their interface. The predominant reason for such behaviour could be the difference in the nature of the cell wall layers. Along with the differences mentioned above the way, checking failure is taking place in wood could also be related to the elastic properties of wood. When two cell wall layers differ in their elastic nature, one that is more elastic will bend more easily as compared to the less elastic layer when force is applied to them (Jackson *et al*., 2004). The middle lamella and primary wall are more elastic in nature compared to the S_1 layer, as they have higher content of the amorphous matrix and very few to no cellulose microfibrils (Cousins, 1974). The difference in the cellular microfibril orientation (Niklas, 1992) could result in the cml and S_1 delaminating from each other during drying stresses leading to check surfaces in wood cells.

2.4 Summary

Intra-ring checking generally occurs as intercell failure where the split is seen to be at the cml/S_1 boundary. The nature of the cell wall is the main reason for the failure to occur this way in the radiata wood. The difference in the cellulose microfibril orientation, lignin content and to some extent the non-pectic polysaccharide content in the cell wall layers are the possible factors exerting their influence on the location and formation of checks. The following chapter will look at the role of the tracheid dimensions and their influence on checking in radiata pine wood.

Chapter Three

Tracheid dimensions and their influence on checking

3.1 Introduction

Radiata pine is an important plantation species of New Zealand, however, its tendency to check while drying leads to significant loss of value of its timber. Over the past five decades considerable research effort has been directed towards means of preventing or minimizing drying degrade in timber by modifying drying schedules, chemical treatments and surface tension changes by using surfactants and sap replacement methods, bulking agents, pre-treatments and surface coatings (Ilic, 1999b and references therein). In chapter two, the location of checking was determined; however, the cause of the split was not determined. The study being discussed in this chapter was designed to understand the structure and composition of the tracheids of checked wood, in order to determine if there were any differences in the tracheids of the checked wood compared to the non-checked wood. Light microscopy and scanning electron microscopy were used to investigate the signs of stress development in the checked wood, to provide some insight into the behaviour of checked wood tracheids and the factors that could lead to checking.

3.2 Material and Methods

3.2.1 Experimental Design

A comparative study was conducted of the tracheids of checked and non-checked wood. For this study, two samples were used from each of the severe, moderate and non-checked wood categories (refer to section 2.2.1 and Figure. 2.1). In order to minimize the

tracheid variations that occur across the growth ring the samples were collected from growth ring 7, and mostly from the middle region of the earlywood and latewood. A large number of tracheids were used for this study to compensate for the variation in cell size. We used both light microscopy and scanning electron microscopy for the study.

3.2.2 Examination of the checks seen in the radiata pine discs

The samples of checks were examined using Scanning electron microscope and light microscope. The sample preparation for the scanning electron microscope is the same as described in section 2.2.2. For light microscopy wax, stained sections of the checks and the same slides were used to study the tracheid dimensions as well. The preparation of the permanent slides is being described in the sections below.

3.2.3 Samples for tracheid dimensions study

Checks along with surrounding wood tissue were removed from growth ring seven of the oven-dried discs of radiata wood. Checks from the two severe checked discs, six checks from two moderate checked discs, and four samples of wood tissue from two non-checked discs, approximately from the same locations on the growth ring as the checked samples were used for this study. Wax sections were cut, stained with safranin, and counter stained with fast green (Johansen, 1940, Chaffey, 2002a) to give a better contrast and visibility of the tracheids and their cell wall layers in the section. This allowed easy and more accurate image analysis of the sections.

3.2.3.1 Preparation of permanent slides for the study of tracheid dimensions

Tissues of the excised samples were fixed with 4% formaldehyde (section 6.2.6.1). The samples were dehydrated using a graded ethanol and tertiary butyl alcohol series (TBA; BDH, Poole, England). The ethanol series consisted of 10, 20, 30, 50, 70, 80, 90 and 100% ethanol (MERCK, Germany) and the TBA series consisted of five grades. These were made up as follows in parts by volume: grade 1: 40 mL distilled water, 10mL TBA and 50 mL ethanol (95%); grade 2: 30 mL distilled water, 20mL TBA

and 50mL ethanol (95%); grade 3: 15 mL distilled water, 35mL TBA and 50 mL ethanol (95%); grade 4: 50mL TBA and 50 mL ethanol (95%); grade 5: 75mL TBA and 25mL absolute ethanol (MERCK, Germany). This is then followed by two changes in 100% TBA for 1 hour each, the third and the last change of TBA for approximately 12 hours. The last three changes of TBA are carried out in a 40°C oven (Hearson, WillowWalk, London) as TBA freezes at 24°C.

The samples prepared were then infiltrated with wax. The first change was done with 50/50 TBA/ liquid paraffin. This is followed by three changes in molten embedding paraffin wax for 24 hrs at 60°C under vacuum (Hearson, WillowWalk, London). The samples were then embedded into plastic moulds (Simport, Canada) that were filled with liquid Paraplast (Oxford Labware, St. Louis, USA) with the help of a mould station (Tissue-Tek dispensing console, Miles Scientific, USA). The mould was then placed on cold stage (Tissue-Tek cryo console, Miles Scientific, USA). Once hard, the wax blocks were trimmed and 10 μm thick sections were cut on the rotary microtome (Reichert Jung, Heidelberg, W. Germany). The sections were transferred onto clean slides (Marienfeld, Germany). The slides were then left on warming plate for a few minutes to melt the wax and stick the section to the slide. Additional slides were prepared by Med-Lab (Christchurch).

3.2.3.2 Dewaxing and staining of the slides for the study of tracheid dimensions

The sections were dewaxed and stained. For dewaxing, the slides were taken through a xylol series (100% xylol for 1 minute followed by two 5 minute changes). The slides were then taken through different grades of ethanol (50: 50, 100 % xylol: 100% ethanol; 100%, 95%, 80%, and 70 % ethanol all for 1 minute each) and left to stain in safranin (1% safranin in 70% ethanol) (Sigma Chemical Co., USA) for 24 hours. After rinsing in distilled water, the slides were then transferred for 10 seconds in 95% ethanol and 0.5% picric acid. They were then transferred to 100% ethanol and ammonium hydroxide (Sigma Aldrich, St. Louis, USA) (4 drops in 100 mL of ethanol) for 10 seconds, and rinsed in 100% ethanol for 10 seconds before they were counter stained with 0.05% fast green (Aldrich Chemical Co., USA) (in 95% ethanol) stained for 10

seconds. The stained slides were then taken through series of ethanol (100% ethanol 3 changes of 5 seconds each) and xylol (1 change of 50:50 100% xylol: 100% ethanol; followed by 3 changes of 100% xylol for 3 seconds each). The sections were mounted with DPX as the mounting media and cover slipped (BioLab Scientific).

3.2.4 Images for the tracheid dimension study

The tracheid cells in the samples were observed with a 10x objective for tracheid dimension analysis and a 40x objective for cell wall thickness of the tracheid using an Olympus BH-2 microscope. To decrease the variation in data caused by the seasonal continuum of the tracheid structure images were collected from the central portion of the earlywood or the latewood. Images were collected using a Cool Snap CCD camera (RS Photometrics) at standardized lighting and exposure conditions.

3.2.5 Image analysis for tracheid dimensions

Earlywood and latewood tracheid dimensions were measured using the safranin-fast-green stained sections. The strong staining of the cell wall made it possible to trace out cell boundaries using the trace function of the Image-Pro Plus software package (MediaCybernetics Inc., Maryland USA). Rays and partial cells were removed by using the selection tool of the Image-Pro. Pits were removed from the data set based on their small size. Tracheid cross sectional area, radial width and tangential width were calculated with the Image-Pro Plus software using the cleaned trace image. Tracheid lumens were selected as the bright areas in the image and measured by the software. Partial lumens, pits, and multiple lumens fused at pits were omitted by using the toggle off function of Image Pro. The cleaned image was analysed for lumen cross sectional area, radial width and tangential width with the Image Pro software.

3.2.6 Image analysis for tracheid cell wall thickness

Cell wall thickness and middle lamella thickness were measured using enlarged images of the ruthenium red stained sections (method described in section 4.2.4.2) and the length function of the Image Pro software package.

3.2.7 Samples for tracheid dimensions study using FibreLab

Wood samples were collected from the seventh growth ring and dissected into earlywood and latewood prior to maceration. The wood pieces were cut approximately 2x 2mm x 3cm long (similar to match sticks). They were then macerated separately and measured for various tracheid dimensions using FibreLab ^{TM}(Metso Automation).

3.2.7.1 Macerations of the samples

The method for the preparation that was followed was as per Franklin's technique as described in Chafey (2002a). The individual sample pieces were put into test tubes and to each test tube 3 mL of 50% hydrogen peroxide and 3 mL of glacial acetic acid (BioLab, Clayton, Vicotria, Australia) was added, and left to incubate in a fume cupboard at 90°C (in a water bath) for 4 hours or till the samples of wood were bleached (i.e. the pieces were whitish in colour). The macerating solution was carefully decanted and 30 to 40 mL of distilled water was gently added to the tubes to wash out any remaining macerating solution. This was repeated 3-4 times. Finally, 15 mL of distilled water was added to the tube and shaken vigorously to break up the wood into long thread like tracheids or fibres. The samples could then be analysed or stored in jars filled with distilled water for many months at 4°C.

3.2.7.2 FibreLab analysis of macerated wood

FibreLab ^{TM} (Metso Automation) was used to collect measurements for tracheid length, tracheid width and cell wall thickness and to calculate cross sectional area and tracheid volume following the protocols from the FibreLab ^{TM} manual. Data were weighted to remove fibrils and cell wall fragments. The histogram of tracheid length was bimodal with the debris mostly confined to the smaller peak. The data points within this peak were subtracted from the data set, in order to ensure that primarily measurements of intact tracheids were only considered.

3.3 Results and Discussion

3.3.1 The intra-ring checks were the widest in the earlywood region

The intra-ring checks spanned the earlywood and were seen to terminate in or at the latewood region of the growth ring (Figure.1.13). Almost all the checks were seen in the earlywood region and no checks were observed in the latewood region of the wood. The check frequently begins in the weaker earlywood and very rarely extends across the latewood region (Chafe, 1995; Cown *et al*., 2003). The checks were the widest in the earlywood region (Figure. 3.1) indicating that the nature of the earlywood tracheids is more conducive for check formation. The earlywood is considered to develop checks more easily as it is weaker than latewood and is not able to transfer its stress as at is restrained by the stronger latewood (Chafe, 1995).

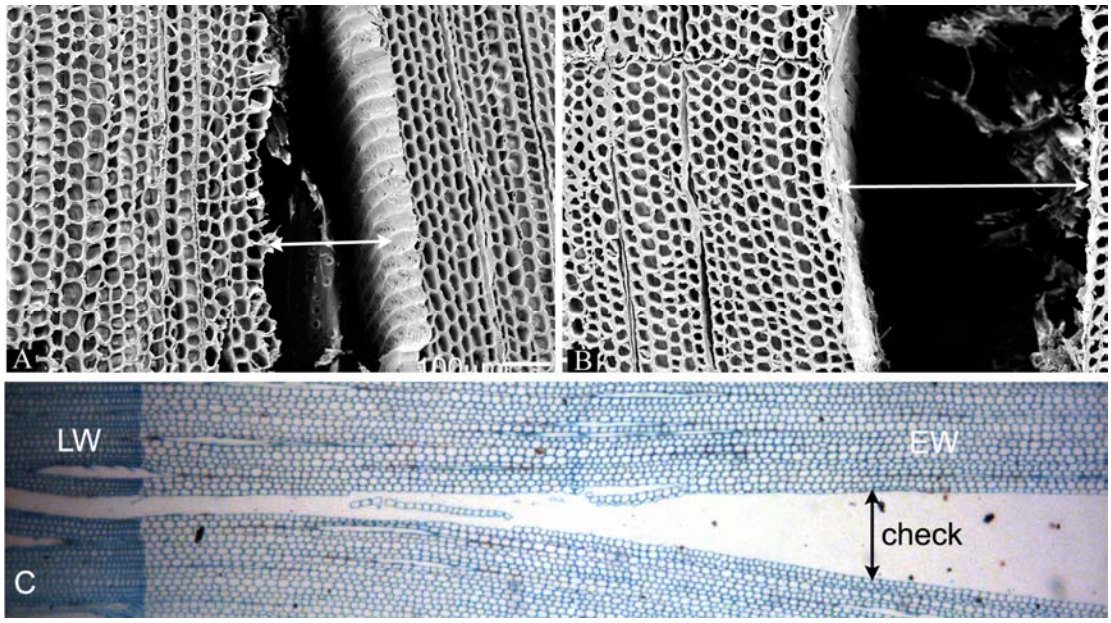


Figure. 3.1 *The checks were the widest in the earlywood region of the oven-dried discs of the radiata pine wood.*

3.3.2 Collapsed cells were seen along and distant from the checks

Collapsed tracheids were seen in the earlywood that had developed checks (Figure. 3.2). Similarly, collapsed rays and epithelium cells of resin canals were also observed to be associated with checks (Figures. 5.1 and 5.3). Some of the collapsed cells were observed along the edge (Figure. 3.2 B and D) of the checks, though most of the collapsed cells seem to occur some distance away from the main split. The deformed tracheids were seen frequently around the middle portion of the earlywood region between adjacent checks (shown by arrows in Figure. 3.2 A, C, E and F). The location of the cells leads to the conclusion that these cells had not collapsed, but had been crushed by the release of energy that took place as the split developed in the wood. As pointed out by Chafe (1995) the earlywood is restrained by the stronger latewood bands on either side. Hence, as it stressed during the drying process it is unable to transfer its stress to the surface and relax. The inevitable stress relaxation occurs instead through the formation of cracks within the earlywood i.e. as checks. A second hypothesis is that collapse of cells preceded the split in the wood as the check developed. Yang (1998) reported that the internal checks may be initiated by collapse in the early stages of drying, and that their later enlargement could be attributed to the drying stresses (like shrinkage as this study suggests). The checks could be the possible location of release of the cross-grain tension stresses, which are created by the initial caving in of the cells (Bariska, 1992). These observations were common in both the severe and moderate checked discs. No collapsed cells were observed in the non-checked wood.

3.3.2.1 The role of collapse in check formation

There is significant relationship between collapse and checking (Ilic, 1999a). Intra-ring checks can be induced from collapse of cells that is usually seen to occur in the earlywood regions (Innes, 1996b). According to Ilic (1999a), collapse occurs during drying as water is removed from highly impermeable wood cells, which become distorted because of high tensile forces generated in the lumen water. The earlywood cell walls of low rigidity crumble into the lumen space thus producing the distorted cells.

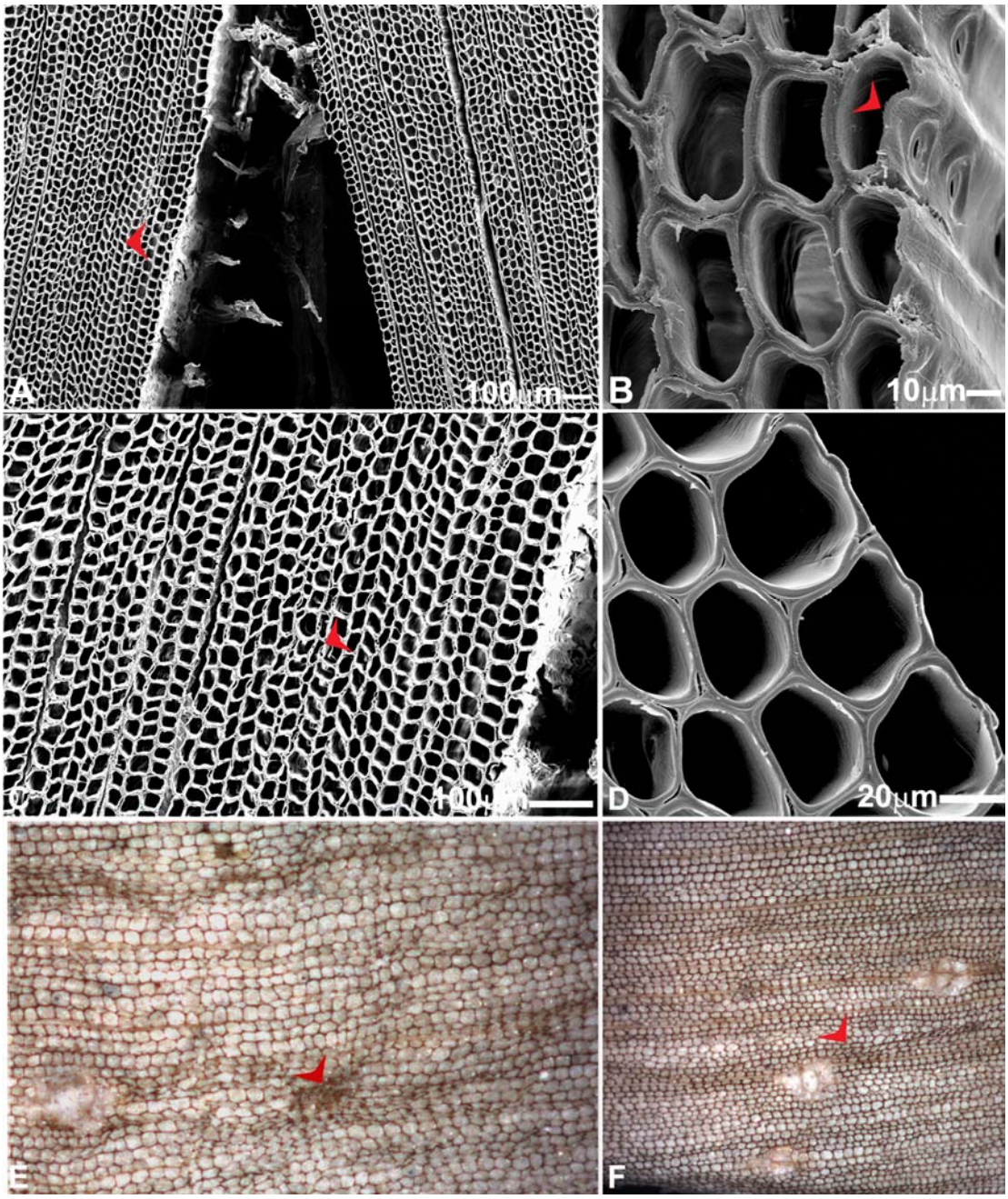


Figure. 3.2 *Collapsed tracheids were seen in checked wood. Tracheids that were distorted in their radial dimension were often found to be distant from the crack of the check (A, C, E and F, arrowheads). Collapsed tracheids were seen adjacent to the crack (A). The distortion of tracheids could also be minimal along the edge of the crack (B).*

Panshin and deZeeuw (1970) attempted to explain collapse based on liquid tension in the cell cavities and compressive stresses exerted on the cell walls during drying. As the water is removed from the cell lumen, it exerts tension on the cell wall layers, this kind of tension can be set up only when the tracheid lumens are completely filled with water. In the latter case the collapse occurs when the compressive stresses developed during drying exceed the compressive strength of wood, as a result the cell wall layers of a tracheid collapse into its lumen. Similar theories of collapse in wood have been put forth by Booker and Sell (1998). They state that the cell walls have to be able to withstand collapse under hydrostatic tension forces that act perpendicular to the cell walls of wood (Figure. 3.3A). Consider two adjacent cells, if one is water filled and the other air or vapour filled (as could be the case during drying of wood), the transverse forces on the cell wall between them are no longer equal and opposite. The transverse force on the cell wall tends to push the cell walls into the lumen, causing cell collapse (Figure 3.3B).

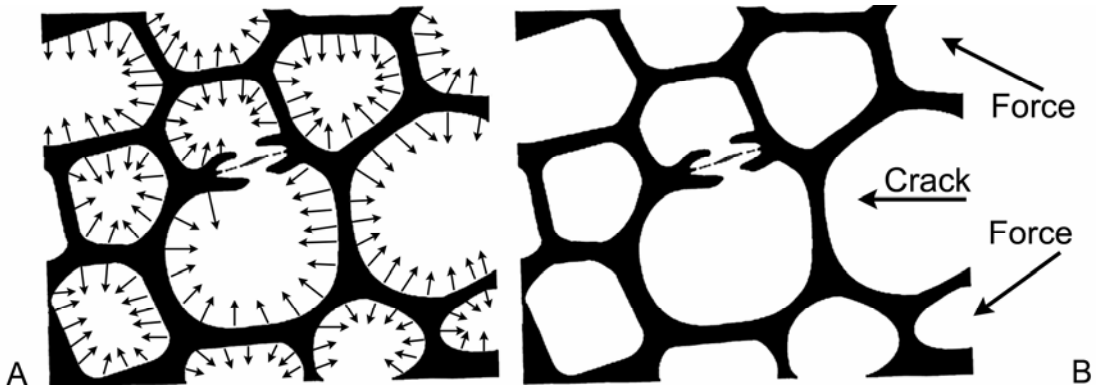


Figure. 3.3 *The schematic diagrams are an attempt to explain the mechanisms that may take place during the process of internal checking as the wood dries (Booker & Sell, 1998).*

The above theories suggest an explanation for the reason for the heartwood to rarely show checks on drying. The sapwood of radiata pine has high moisture content, typically 120-180%, and in contrast, the heartwood has only a moisture content of 40%, and only few of its cells are water filled (Kininmoth & Whitehouse, 1991; Bariska, 1992; Simpson *et al*., 2002). Consequently, there is less differential water tension in heartwood

compared to sapwood and hence we see more collapse and checks in sapwood than heartwood. In addition the sapwood could be more prone to collapse and checking as the sapwood has higher ratio of earlywood and also the cell walls are thinner and so they are more likely to give into the tension forces compared to the more resistant heartwood (Simpson *et al*., 2002),.

Collapse severity is known to depend on drying temperature and relative humidity particularly in the early drying stages of drying (Greenhill & Dadswell, 1938; Ellwood, 1952; Kaunman, 1958). During high temperature, drying moisture gradient develop in the timber and is affected by the fibre saturation point. When the cell walls of wood cells are fully saturated with hygroscopic moisture and there is no free water in the cell lumen then this moisture content is referred to as 'fibre saturation point' (Kininmoth & Whitehouse, 1991). Collapse can start in timber well above fibre saturation point of wood and take place in the hygroscopic range (Bariska, 1992).

Checks can appear above the fibre saturation point due to the differential stresses in the earlywood and latewood causing splitting to occur in the weaker earlywood region (McCurdy & Keey, 1999). Innes (1996a; b) suggested that collapse could be eliminated from wood if it was dried below 'collapse threshold temperature'. Ilic (1999a) critiqued the work stating that though the severity of collapse is related to drying temperature, the experiments conducted by Innes (1996a; b) were not conclusive enough to indicate threshold temperature and the concept needed further definition.

3.3.3 Shrinkage of wood could also be involved in check formation

The presence of collapse does not necessarily indicate internal checking; California redwood and Western red cedar for example collapse frequently but rarely show intra-ring checks (Cown *et al*., 2003). Therefore, there is a possibility that checks could be developing in wood by mechanisms other then collapse, such as shrinkage. Stress induced by shrinkage also could result from water tension superimposed on the cell wall layers (Hunter, 2001). This could stress the cells leading to check formation. Some earlier studies do suggest that shrinkage plays a role in checking (Ilic & Hillis, 1986; Chafe, 1986, 1994a, b; Ilic, 1999a, b).

Shrinkage differs from collapse, as shrinkage is caused by cell walls drawing closer as water dries out from the wall layers, while in collapse the walls of the cell are actually pulled together causing the cell to cave in as water is removed during drying (Panshin & deZeeuw, 1970). Wood undergoes anisotropic shrinkage on drying (Barber & Meylan, 1964; Yamamoto, 1999; Pang, 2002). During drying moisture is lost from the wood surface more easily as compared to the centre, this leads to the development of moisture content gradient that results in uneven shrinkage (Pang, 2002). The outer layers try to shrink before the inner core, and when the stress between the layers exceeds the yield stress of the wood checking takes place (McCurdy & Keey, 1999). Both the external and internal shrinkage tend to be positively correlated to one another (Chafe, 1994b). The prime determinant therefore is mainly the predisposition of the material towards shrinkage (Chafe, 1994b), and checking as well.

The extent of shrinkage differs in the three different anisotropic directions namely longitudinal, radial and tangential directions. This highly variable shrinkage property worsens wood deformations with drying (Pang, 2002), and checks are known to form in wood in such local regions of high differential shrinkages (Bisset & Ellwood, 1951).

In order to analyse the influence of wood shrinkage behaviour on lumber deformations during drying, one need to consider the interaction between earlywood and latewood (Pang, 2002). The earlywood shrinks more in the longitudinal direction and less in the tangential direction (Kininmoth & Whitehouse, 1991; Pang, 2002). According to Pang (2002) the earlywood shrinks more than the latewood, thus it is stretched (positive stress) by the latewood, while the latewood in the meantime is compressed (negative stress). This will lead to the wood bending downwards. In the tangential direction, the earlywood is compressed and latewood is stretched, this will lead to an upward bending. This kind of net movement that takes place in wood during drying could cause stresses to develop in the wood leading to shearing amongst the cell wall layers and possible splitting and development of check (Figure. 3.4).

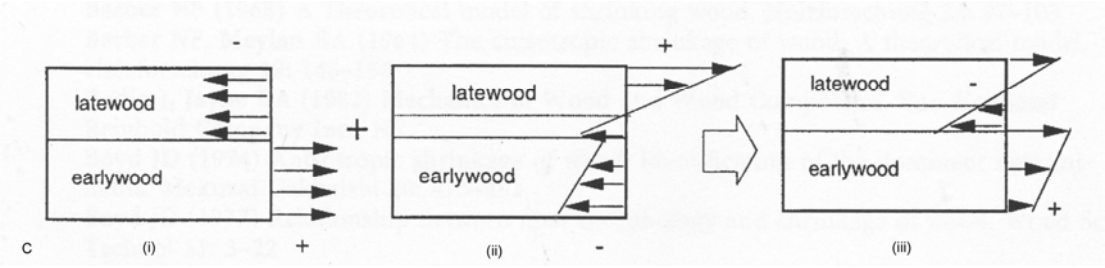


Figure. 3.4 *The earlywood shrinks more than the latewood, thus it is stretched by the latewood, while the latewood in the meantime is compressed. This will lead to the wood bending downwards. In the tangential direction, the earlywood is compressed and latewood is stretched, this will lead to an upward bending. This net movement in drying wood can cause stresses to develop in the wood leading to shearing and splitting amongst the cell wall layers and possible development of check (Pang, 2002).*

3.3.4 The checked wood tracheids had wider radial dimension

Tracheids were examined by light microscopy and then evaluated with image analysis (Table. 3.1). The tracheids in checked wood were wider than in non-checked wood. In checked wood, the earlywood tracheids were on an average 19.7% wider in the radial dimension and 9.7% wider in the tangential dimension than in the non-checked wood. The cross sectional area was 32% bigger than in the non-checked wood.

The FibreLab^{TM} analysis of the macerated fibres showed similar trends. The tracheids from checked wood had larger cross sectional area and fibre width compared to the non-checked wood samples (Table. 3.2). These results indicate that the checked wood tracheids had a different shape. As the cells were wider in the radial dimension, it seems likely that these cells could deform more easily when subjected to drying stresses, for example, the longitudinal shrinkage stresses would be pronounced in these types of earlywood cells, so that they would give in more easily to the stress and possibly checking.

Some of the previous studies state that the tracheids of radiata pine wood tend to be broader in their radial dimension (Nyakuengama *et al*., 1999, Shelbourne *et al*., 1997). Our average value of 43.15 μm is at the upper limit of the radial dimensions reported (Shelbourne *et al*., 1997). During the evaluation of the FibreLab^{TM} data, it was not possible to distinguish radial width from tangential width as the wood structure was

destroyed by maceration. In addition, the tracheid dimensions measured with FibreLab™ were consistently larger than those measured with image analysis (compare Tables. 3.1 and 3.2). The differences reported here were similar to those measured by others and were consistent with the lower resolution of the optics in the FibreLab™ compared with the image analysis done on the images collected from light microscopy. Furthermore, the hydrated state of the macerated fibres could also influence the accuracy of the measurement (Richardson *et al*., 2003).The data obtained from image analysis was more accurate than the FibreLab™ data .

According to Wang *et al*., 2003, larger diameter tracheids have high rates of xylem conductivity, and large tracheids are more vulnerable to cavitation (upon vaporisation of their water content). The earlywood has higher xylem conductivity, greater vulnerability to cavitation than latewood, as latewood is composed of narrower tracheids with thicker walls, and this may serve as a protection against cavitation (Wang & Aitken, 2001). So it seems it is not just the shape but other factors like cell wall thickness that need to be considered as the properties of tracheids affecting check formation.

3.3.5 Lumen dimensions also varied with checking

Tracheid lumens, like the tracheids had a larger cross sectional area in the checked wood (Table 3). The lumen dimensions did not proportionally match the changes with the tracheid width, indicating differences in cell wall thickness.

During high temperature drying of wood as per Ping and Lianbai (2003) three types of moisture transport of water are seen: i) liquid movements through cell lumens and pits above fibre saturation point as a consequence of capillary action between liquid and gas phases inside the capillary; ii) bound water diffusion through the cell-walls below fibre saturation point due to moisture gradients across the cell-wall; iii) water vapour that moves towards the surface of wood under a partial vapour pressure gradient through the whole moisture content range during drying. In the case of high temperature drying, the moisture content is initially high; as a result a high moisture gradient,

Wood characteristic	Severe checking	Moderate checking	No checking
Fibre width in radial axis(µm)			
Earlywood	43.15	43.15	36.02
Latewood	31.90	35.65	28.14
Fibre width in tangential axis(µm)			
Earlywood	35.65	36.40	33.40
Latewood	33.40	34.15	32.64
Fibre width ratio (radial/tangential)			
Earlywood	1.22	1.20	1.10
Latewood	0.98	1.06	0.91
Fibre cross sectional area(μm^2)			
Earlywood	1134.6	1166.3	903.66
Latewood	847.34	888.73	709.35
Lumen cross sectional area(μm^2)			
Earlywood	754.27	859.02	622.41
Latewood	430.85	460.42	365.10
Lumen width in radial axis(µm)			
Earlywood	36.77	37.90	30.39
Latewood	23.64	25.52	20.64
Lumen width in tangential axis(µm)			
Earlywood	27.77	29.64	26.64
Latewood	22.89	23.26	22.51
Lumen width ratio (radial/tangential)			
Earlywood	1.37	1.28	1.17
Latewood	1.07	1.12	0.96
Cell wall thickness in radial axis(µm)			
Earlywood	2.70	2.54	2.89
Latewood	5.33	5.70	5.11
Cell wall thickness in tangential axis(µm)			
Earlywood	2.56	2.12	2.53
Latewood	5.48	6.27	6.22
Lignin proportion in cell wall			
Earlywood	0.094	0.147	0.230
Latewood	0.3121	0.3718	0.6838
Middle lamella width in radial axis(µm)			
Earlywood	0.95	0.96	0.96
Latewood	1.14	1.14	0.87
Middle lamella width in tangential axis			
Earlywood*	1.41	1.33	1.43
Latewood	1.88	2.09	1.45

*mixed groupings –severe and no check formed one group, severe and moderate a second group.

Table. 3.1. *Image analysis of wood samples with intra-ring checking showed that they could be separated from check-free samples using a range of tracheid features. Differences between the medians (Kruskal-Wallis statistic) are indicated by colour. Samples that were not different have the same colour. An expanded version of this table is presented in Appendix 1.*

Wood characteristic	Severe checking	Moderate checking	No checking
tracheid length (mm)			
Earlywood	2.74	2.74	2.72
Latewood	2.97	2.98	2.52
tracheid width (μm)			
Earlywood	49.06	49.80	46.53
Latewood	47.97	47.11	42.51
tracheid cross sectional area(μm^2)			
Earlywood	1512.8	1543.8	1362.4
Latewood	1481.7	1451.5	1169.7
tracheid volume(mm^3)			
Earlywood	4.01	4.17	3.70
Latewood	4.25	4.19	2.94

Table. 3.2. *Tracheid dimensions measured and calculated using FibreLab™. Wood samples that underwent intra-ring checking upon oven drying had differences in their cells, cell walls and lumens as reported below. Kruskal-Walis statistic was used to detect differences between the groups. Groups are indicated by colour. When there was no difference between samples, they were given the same colour. Additional information is given in an expanded version of this table in Appendix 3.*

Key : indicates the largest sample the intermediate, and the smallest.

can occur as water flows towards the surface of the wood due to the liquid pressure (Ping & Lianbai, 2003). The pressure gradient has developed due to the capillary action between liquid and gas phases within cell lumens of the wood (Ping & Lianbai, 2003). Hence, it seems that when the moisture content in wood is below fibre saturation point, the moisture movement is mainly by diffusion of bound water and water vapour under moisture-concentration gradient, whereas when moisture content is above fibre saturation point, the moisture movement is by permeability of free water and water vapour under capillary pressure and partial water vapour gradient. When the surface tension of the water in these capillaries increases beyond the strength of the walls, it results in distortion of the cell wall and collapse of the cell (Bariska, 1992). Hence, it seems that lumen size can influence the changes in the moisture gradient as the wood dries and in turn affect

checking in wood by the above mentioned mechanisms. Thus, a tool that measured cell lumen dimensions could possibly detect check prone wood.

3.3.6 Cell walls were thinner in the radial axis in checked wood

The average cell wall thickness varied from 2.12 to 2.89 μm in earlywood. This is within the range of cell wall thickness reported for radiata pine (Shelbourne *et al.,* 1997; Kibblewhite & Bailey, 1988; Kibblewhite & Evans, 2001). The checked wood had thinner cell walls in the radial dimension compared to the non-checked wood, but not necessarily the tangential cell wall layers, where the severe checked wood had cell walls even thicker than the non-checked wood sample. However, the moderate checked wood did show overall thin cell wall layers both in the radial and tangential direction (Table. 3.1). The cell walls in latewood were much thicker for all samples. FibreLab^{TM} was not able to accurately measure cell wall thickness (Appendix 1). Hence, a comparison could not be done between the results of the two studies conducted.

Checks seem to appear in wood due to differences in the earlywood and latewood cell wall thickness and size of tracheids that can cause splits to initiate in the weaker and less stiff earlywood region (Chafe, 1994b). One of the primary reasons for the latewood not to develop checks could be the cell wall thickness. The thick cell walls of latewood can possibly withstand the differential moisture gradients, and compressive forces that are generated during drying and resist collapse and checking. As per Chafe (1994b), in collapse susceptible material the thickness of the cell wall is critical in resisting liquid-tension forces associated with drying. Thus, the thin walled cells (like the thin walled cells of the checked wood) collapse and shrink more easily. If the differences between the radial and the tangential thickness of the cell walls are large, then the cells will have high radial shrinkage (Barber & Meylan, 1964). This could lead to differential shrinkage and in some cases checking.

3.3.7 A higher tracheid width/cell wall thickness ratio was associated with checking.

From the discussion above (section 3.3.2.1), it seems that collapse of the tracheids could be one of the primary factors that can cause checking in wood as it dries. Prediction of collapse could possibly help in indicating susceptibility of wood to checking. The susceptibility of tracheids was using the tracheid width/cell wall thickness ratio (Kibblewhite *et al*., 2000). A wide tracheid with a thin cell wall would have a higher ratio and be more likely to undergo collapse. This ratio, called the tracheid collapse potential, comes from the geometric components involved in material deformation. The ability of a material to withstand a deforming force is determined by its length, width and modulus of elasticity. For a tracheid resisting collapse during drying these, correspond to the tracheid width, the thickness of the cell wall and the cellulose microfibril angle (Jackson & Nair, 2003).

Intra-ring checking is likely to happen in cells due to collapse and due to differential anisotropic shrinkage especially in the longitudinal direction (section 3.3.3). Light as well as scanning electron microscopy showed that that the cells were deformed mostly in the longitudinal direction along the radial wall. For this reason, the tracheid width and cell wall thickness along the radial axis of the tracheid are important in determining collapse (S. Pang in pers comm). It was found the collapse potential for the radial dimension to be higher for earlywood from the checked wood samples (Table 3.3), while all latewood tracheids irrespective of checked or non-checked wood had a low collapse potential. Wilson and White (1984) pointed out that the tangential diameter in the earlywood and latewood is generally constant, but the radial diameter decreases and the cell wall thickness increases. In case of the checked wood the radial diameter is high, however, the cell wall thickness is not increased. Hence, it seems that the earlywood is more prone to checking and latewood is resistant to checking. From these results, it seems that radial collapse potential could be a good indicator for the tendency of wood to check.

Radial tracheid collapse potential			
Wood region	Severe checked	Moderate checked	Non-checked
Earlywood	15.90	16.34	12.05
Latewood	5.94	6.16	5.63

Table. 3.3 *Radial tracheid collapse potential was calculated for the radial dimension (tracheid width/cell wall thickness in the radial direction) was highest for samples that had intra-ring checking. Calculations used average values (Table 2 and Appendix 1).*

3.3.8 Tracheid length did not vary in the earlywood

There was no appreciable difference between the length of the tracheids in the checked and the non-checked wood (Table 3.1). In fact, the checked wood had slightly longer tracheids compared to the non-checked wood. The latewood tracheids were longer than the earlywood tracheids in the checked wood, but shorter than the earlywood tracheids in the non-checked wood. The lengths reported here are within the range of those reported earlier for radiata pine suggesting that tracheid lengths were not atypical in the checked wood (Richardson *et al*., 2003; Kibblewhite & Uprichard, 1996; Matsumura & Butterfield, 2001).

The earlywood tracheids were generally shorter than the latewood tracheids of the same growth ring and this is one of the reasons for their reduced stiffness and strength (Panshin & deZeeuw, 1980). Latewood tracheids exhibit greater strength and stiffness irrespective of tree height or juvenility as per Mott *et al*., (2002), so it seems that the latewood is better equipped to withstand the differential forces that are generated in wood during drying without developing intra-ring checks.

3.4 Summary

The results presented in the current chapter and from the previous studies, it is quite evident that shrinkage and collapse play an important role during drying of wood. The behaviour of wood while drying in turn can influence the intra-ring checking. Both collapse and shrinkage in wood are affected by factors of wood structure such as the

properties of earlywood and latewood and the moisture content of wood and as well as by the drying conditions.

Collapse alone can explain nearly 50% of variability displayed by checking in wood (Ilic, 1994a). The development of the intra-ring checks is consistent with collapse occurring in the low strength earlywood region of the wood that is restrained by the high strength denser latewood (Ilic, 1999b). The shrinkage anisotropy can also influence the development of checks and is dependant significantly on the mechanical interaction between earlywood and latewood (Watanabe *et al*., 1998). However, the most dominant factor is the tracheid structure that constitutes the bulk of the wood. The combination of smaller cell lumen and thicker cell walls ensured that the cells were able to withstand the forces of stress generated during drying (Simpson *et al*., 2002) and resist checking.

If the geometry of the tracheid is asymmetric, then an asymmetric distribution of stresses will be generated that can lead to checking in wood. The intensity of the checking will most likely be influenced by the tracheid width/cell wall thickness ratio. The higher the ratio the more likely the wood will check on drying. There was variability in the tracheid dimensions along the radial direction, and any change here will influence the drying stresses being generated in the wood, as the heat conductivity of the wood is greater in the radial direction than the tangential (Kollmann & Côté, 1968). Hence, the radial dimensions seem to be most crucial for checking. The process of drying and different regimens applied to wood during and before drying does influence checking; however, it cannot mask the predisposition of the wood to check. Therefore, to reduce or to prevent checking in wood not only the drying stresses have to be minimized but also the mature wood has to be modified so that it is able to resist collapse, shrinkage and checking. After looking at the tracheid dimensions investigations were carried out in order to study the chemical constituents of the tracheids of the checked and the non-checked wood. The following chapter will be summarising these finding.

Chapter Four

Lignin levels in cell wall layers play a role in checking.

4.1 Introduction

Intra- ring checking is a highly undesirable and costly wood defect. It causes huge economic losses to the New Zealand forest industry, a 5% reject rate of finished mouldings is equivalent to $\$25/m^3$ loss in log value (Miller, 2004). It is clear from the discussions in chapter two and three, that checking occurs mainly in the earlywood region of wood and is usually caused by the differential stresses experienced by earlywood and latewood regions of the growth ring as the wood dries. Collapse and shrinkage both can play a role in the development of check in wood (as discussed in chapter three). However, the properties of the tracheid were quite important and made the wood either susceptible or resistant to checking (chapter three).

The mechanical properties of timber depend primarily on the properties of the cell wall layers and these in turn by the chemical components that make up the cell wall layers. It has been indicated that the susceptibility of the wood to check could be due to the differences in the chemical composition of the wood (Simpson *et al.*, 2002). This chapter focuses on the results of the histochemical study that was conducted to explore the chemical nature of the cell wall layers of checked wood and non-checked wood. The main aim of the study was to determine any intrinsic differences in the chemical composition of the cell wall layers of checked wood that made it prone to checking.

According to Simpson *et al*. (2002), the chemical composition of the compound middle lamella region can influence the propensity of the wood to check. It was clear from the results of our previous study that checking mainly occurred at the compound middle lamella layer (section 2.3.2), hence, the major chemical constituents of the compound middle lamella like lignin and pectin (section 1.7) were the focus of the study.

Lignin and pectin distributions were observed in the cell wall layers using various histochemical techniques particularly in the compound middle lamella of checked and non-checked wood.

4.2 Material and Methods

4.2.1 Experimental Design

From the results of the earlier study, it was obvious that the checked wood tracheids were different from the non-checked wood tracheids, this led to the development of the current study where study was carried out to see if there were any differences in the composition of the chemicals of the cell wall layers in checked and non-checked wood. In order to study this, the same experimental design as in chapter three (section 3.2.1) was used. We used light microscopy, epifluorescence to visualise the cell wall layers and their different chemical constituents for the study with the help of different histochemical stains.

4.2.2 Examination of the checks seen in radiata pine discs

For light microscopy and epifluorescence microscopy, wax sections of the checks were used. For light microscopy, the slides were observed with the help of Olympus BH-2 microscope with a Coolsnap digital camera (RS Photometrics). The epifluorescence microscopy was carried out with the help of Olympus lX 70 inverted microscope that is attached to mercury lamp. Digital images were collected using a Cool Snap CCD camera (RS Photometrics) using standardised exposure conditions

4.2.3 Sample collection for histochemical study.

The same procedure was followed for sample collection as in section 3.2.2.

4.2.4 Preparation of permanent slides for the histochecmical study of lignin and pectin in the cell wall layers.

Wax sections were cut of the samples of wood and stained with safranin and counter stained with fast green (Johansen, 1940; Chaffey, 2002a) to visualise lignin distribution. Wax sections were also stained with ruthenium red to observe the pectin distribution in the cell walls (Chaffey, 2002a). The microscopy images were further analysed using image analysis software.

4.2.4.1 Preparation of slides for safranin-fast green staining to study lignin distribution using light microscopy.

The same procedure as in section 3.2.2.1 was followed for the preparation of slides for the histochemical study of lignin using safranin-fast green stain.

4.2.4.2 Preparation of permanent slides for the study of lignin using epiflourescence microscopy

Samples were cut and trimmed as per section 3.2.2. The samples were fixed in 4% formaldehyde under vacuum for two hours. The samples were then put through a wax infiltration and embedding protocol (section 3.2.2.1). Transverse or radial sections 10 μm thick were cut, dewaxed (section 3.2.2.1 and 3.2.2.2) and mounted in distilled water and observed with epifluorescence light (excitation wavelength of 280nm) microscopy. An Olympus lX 70 inverted microscope fitted with mercury lamp was used to observe lignin. In the near ultraviolet wavelength, lignin autofluorescence is visible (Scott *et al.,* 1969; Fergus *et al.,* 1969; Fukuzawa & Imagawa, 1981). Digital images of the observations were collected using a Cool Snap CCD camera (RS Photometrics) using standardised exposure conditions.

4.2.4.2 Preparation of permanent slides for the histochemistry of pectin using light microscopy.

The protocol of preparation used for this study was the same as described by Chafey (2002a)**.** Samples were collected and processed as per section 3.2.2. Transverse or radial sections 10 μm thick were cut and dewaxed as per the protocol described in section

3.2.2.1 and 3.2.2.2. The dewaxed samples were then flooded with 1ml ammonium hydroxide (Sigma Aldrich, USA) , in order to delignify the cell wall layers so as to expose the pectin in the cell walls to the ruthenium red. The ammonium hydroxide was then allowed to air dry (for about 2-5 hours). The sections were then washed in distilled water thrice, each wash lasting 15 minutes followed by staining in 0.025% (w/v) aqueous ruthenium red (Aldrich Chemical Co., USA) for 30minutes duration. The excess stain was washed off with distilled water and the slides mounted in DPX mounting medium.

4.2.5 Image analysis

The light epifluorescence and electron micrographs were further analysed for the lignin and pectin distribution using Image Pro Plus (version 4.5 for Windows, Media Cybernetics Inc, USA). The tool of colour base from the software was used for this procedure. The tool allowed the facility of selecting a colour pixel of choice and the area occupied by the pixels of that colour was obtained. In this way, the area occupied by the bright blue area that was displayed only by lignin was calculated. Similarly, the area for pectin was calculated.

4.2.6 Analysis and observations of the homogenous property of wood

Transverse surfaces of the checked wood and non-checked wood were used to study the arrangement of tracheids seen in both types of wood. The observations were made with the help of stereomicroscope (Wild Photomakroskop M400, WILD, Switzerland) that was connected to a Cool Snap CCD camera (RS Photometrics). The images were then closely observed to see the pattern of the tracheids in the checked and non-checked wood.

4.2.7 Binary images used to study the homogeneity of wood

Digital pictures were taken of checked and non-checked oven dried discs of radiata wood using Nikon D1x camera fitted with Nikon 60mm Macro lens. The variations in the colour of the checked and non-checked wood were observed using binary images of the discs. The darkest red colour from the latewood of the disc image was selected, and then used as a mask to binarise the coloured digital image. The

resulting black and white image has black pixels in place of selected reddish colour pixel and the rest of the colours turn out white in the picture. The pictures were binarised using Adobe Photoshop (version 6).

4.3 Results and Discussion

4.3.1 Earlywood and latewood colour differences across radiata wood could be one of the possible indicators of checking

While observing the oven-dried discs of radiata wood, it was noticed that the growth rings in checked wood could be easily distinguished as compared to the non-checked wood. The earlywood/ latewood boundary was more obvious in the checked wood (Figure. 2.1). One of the possible reasons for the increased visibility of the growth rings is due to the changes in the tracheid dimensions within the earlywood and latewood regions of the growth ring (section 1.4.1). However, along with this it was observed that the colour of checked and non-checked wood varied. It is quite possible that the red colouration in the wood was due to the presence of the lignin in the cell wall layers. Latewood was a darker shade of red (higher lignin content) for all groups of wood. Earlywood was whitish in checked wood, but had a more reddish appearance in non-checked wood. When the samples of wood were observed using a stereomicroscope, lignification seemed to vary along the terminal edge of the latewood or the initial earlywood region. Similar observations have been reported earlier (Fukazawa & Imagawa, 1981; Gindl, 2001) where higher lignin concentration generally occurred in the first earlywood cells followed by linear decrease towards the end of the growth ring, and again a variation was seen to increase in terminal latewood where higher lignin content may occur. Furthermore, it was noticed that the non-checked wood appeared more homogenous with an almost even red colouration throughout compared to the checked wood (Figure. 2.1 A and C) giving an impression that there was more even lignification of the cell wall layers in the non checked wood than in the checked wood. In order to explore this further the digital colour pictures of discs were converted to binarised images. The binarised images enhanced the differences in wood colour distribution

making it easier to visualise the distribution of red colour in the wood. The darkest reddish colour of the latewood was selected from the digital photograph of the disc. This was then used as a mask to binarise the coloured image. The resulting black and white image had black pixels in place of the selected reddish colour and white pixels for all remaining colours.

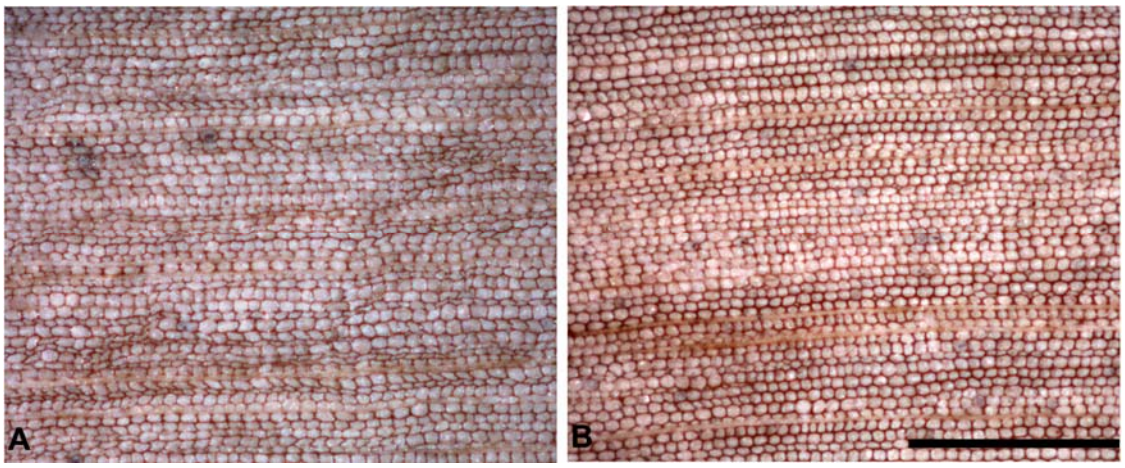


Figure. 4.1 *The checked and non-checked wood samples as seen under a dissectoscope. The earlywood of severely checked samples (A) had less red colouration than that of non-checked wood (B). Scale Bar = 0.5mm*

The binary images of the checked wood have distinct latewood and earlywood bands, compared to the non-checked wood that did not show such distinct demarcation of the earlywood and latewood regions (Figure. 4.2)

The differences in the colouration could be due differential lignification of the cell walls of the wood. There is a possibility that the red colouration could also be due to the incorporation of aldehydes in the lignin of the cell wall. The reddish-brown colour of wood has been reported in loblolly pine cinnamyl alcohol dehydrogenase (CAD) mutant that had high levels of aldehydes (Mackay *et al*., 1996). On the examination of the wood it was observed that the non-checked wood had, a more reddish colour compared the reddish brown colour of checked wood (Figure. 4.1).

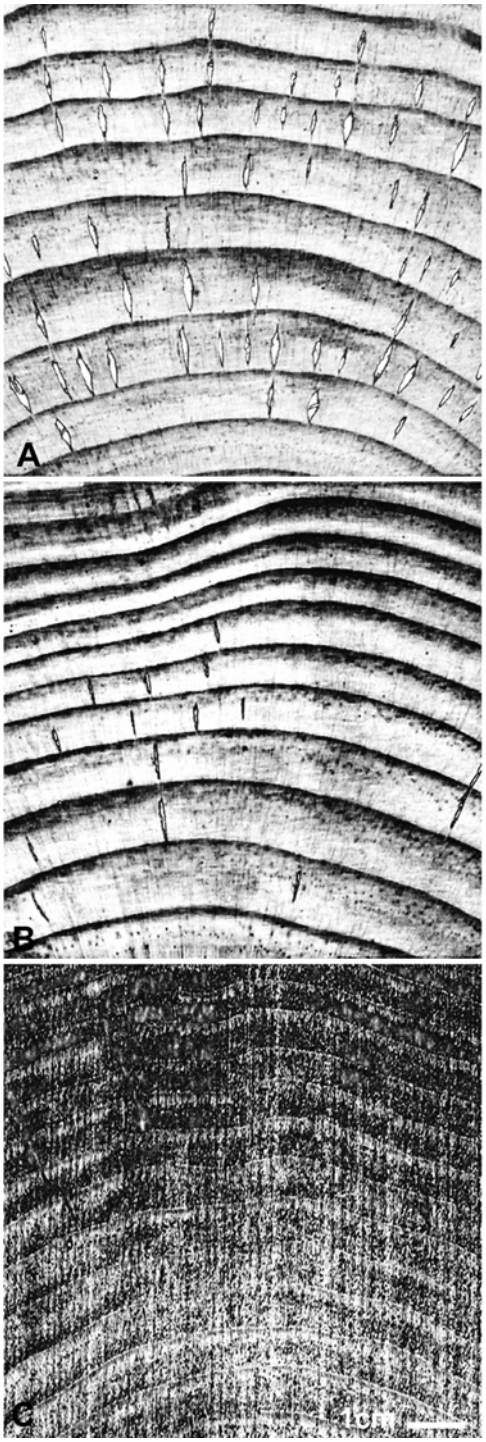


Figure. 4.2 *The binarised images of severely checked (A), moderately checked (B) and non-checked wood (C) are shown here. The dark reddish brown pixels have been replaced with black pixels and all remaining pixels coloured white. The non-checked wood was more homogenous (C) while checked wood contained distinct latewood boundaries (A and B).*

4.3.2 Low lignin in the cell wall layers may lead to checking

When the cell wall layers were viewed using the different microscopy techniques it was observed that, overall the cell walls of checked wood had lower levels of lignin than the non-checked wood.

The earlywood and latewood of both checked and non-checked were compared wood using safranin-fast green as the histochemical dye to visualise lignin and cellulose. Safranin stains lignin red and cellulose greenish blue (Johansen, 1940; Wardrop, 1981). The greater the intensity of red colouration in the cell walls the higher the levels of lignin. In severely checked wood, the lignin level was very low and difficult to distinguish in secondary cell walls of both the earlywood and latewood (Figure. 4.3 compare A, B, C and D to E and F). The non-checked latewood consistently had high levels of lignin and the earlywood had higher levels of lignin as compared to that of the checked wood.

Lignin has a characteristic ultraviolet absorption spectrum with absorbance maxima around 212nm to 280nm. According to Okuyama *et al*., (1998) almost all softwood lignin consists of guaiacyl lignin, which has UV absorption peak at 280nm, hence, 280nm wavelength was used for this study. There is no other major component of the mature wood cell that displays ultraviolet properties in the same spectral range (Scott *et al*., 1969). This property of lignin was exploited to get a more accurate observation of lignin distribution in the cell wall layers of both checked and non-checked wood that might have not been picked up with safranin staining.

In epifluorescence microscopy, the brightness of the fluorescence from the cell wall layers is mainly due to the autofluorescence of the lignin present. The brightness and the intensity of the fluorescence are linearly proportional to the concentration of lignin in the cell wall layers (Kutscha & McOrmond, 1972). In the micrographs the corners of the compound middle lamella stand out with the having the brightest autofluorescence of lignin (Figure. 4.4, seen as light blue colour), followed by the compound middle lamella between the cell corners, while the secondary wall layers appear a dull shade of blue.

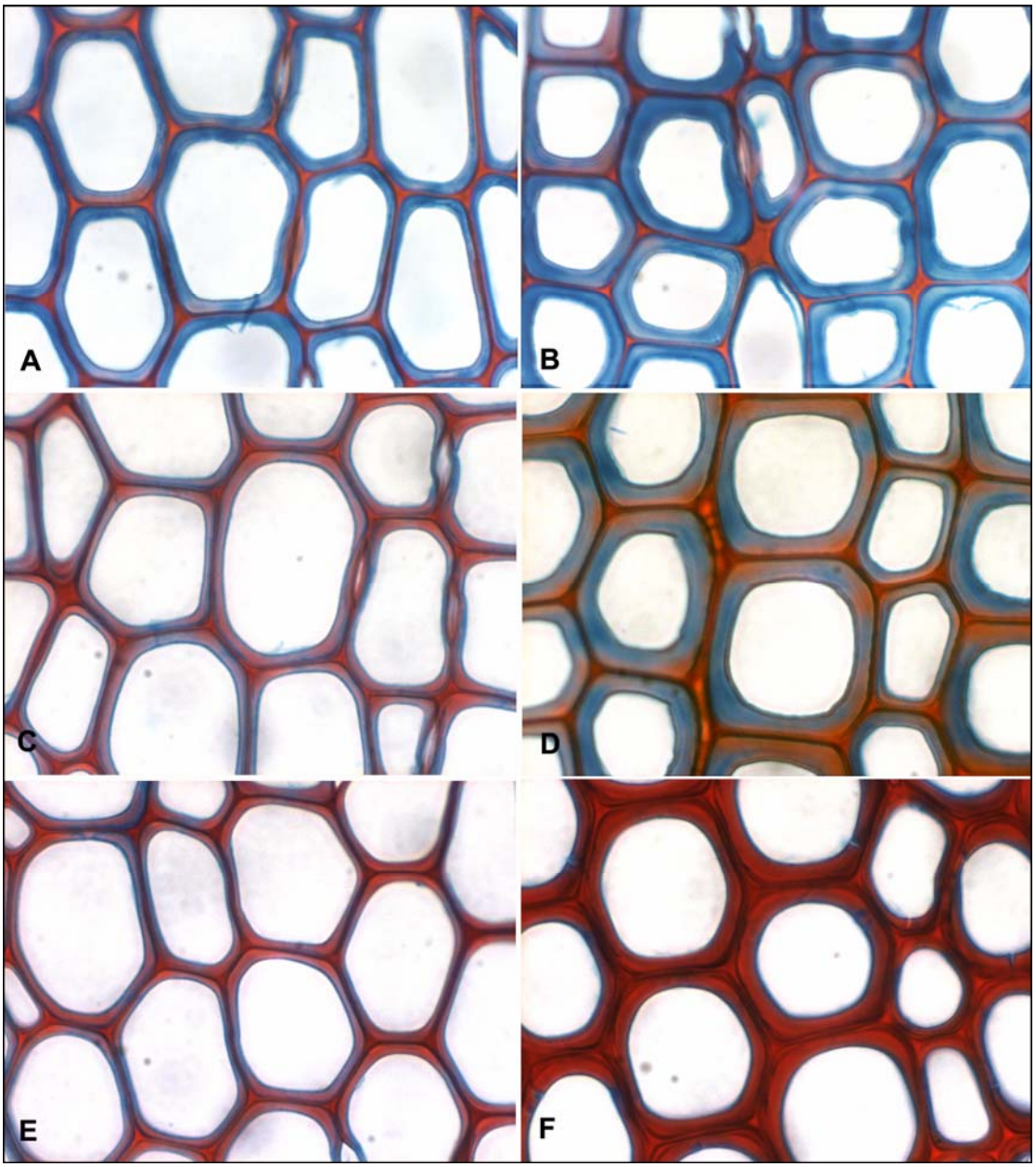


Figure. 4.3 *The light micrographs of wax sections stained with safranin- fast green stain. The lignin stains red with safranin while cellulose is stained blue. In severely checked wood, the lignin level was very low consequently, the red colour is hardly detectable in the cell walls in secondary cell walls of both the earlywood and latewood (compare A, B, C and D to E and F). The non-checked latewood consistently had high levels of lignin (the cell walls stain deep red with safranin) and the earlywood had higher levels of lignin as well compared to that of the checked wood. Light microscopy carried out 100x magnification.*

This indicates that there are higher levels of lignin in the compound middle lamella compared than in the secondary wall layers. This was true for both the checked and non-checked wood and is in agreement with previous studies that show the highest lignin levels were found in the compound middle lamella and the cell corners (Fergus *et al*., 1969; Scott *et al*., 1969; Fromm *et al*., 2003). Comparison of the earlywood of checked wood and non-checked wood displayed results similar to those observed earlier. The checked earlywood had less lignin (Figure. 4.4 compare A and B checked earlywood to C and D non-checked earlywood). There was less lignin in the compound middle lamella in the checked wood in comparison to non-checked wood (Figure. 4.4 C and D depicted by arrows). The S_3 layer also showed more lignin in the non checked wood (Figure. 4.4 C and D depicted by arrowheads in) while lignin in the S_3 layer of the checked wood was almost undetectable.

In order to study the distribution of lignin in the individual cell wall layers, lignin was visualised in potassium permanganate-stained ultra-thin sections using transmission electron microscopy. The dark regions in the micrographs represent lignin (Figure. 4.5). The compound middle lamella was not as darkly stained in severely checked samples compared with the non-checked and moderately checked samples. This indicates that there was a lower concentration of lignin in the compound middle lamella of severely checked wood (Jackson *et al*., 2004). The lignin distribution within the three layers of the secondary wall was also compared. Lignin levels were highest adjacent to the compound middle lamella and rapidly decreased towards the boundary between the S_1 and S_2 layers. Lignin concentration was lowest in the S_2 layer and was elevated again in the S_3 layer (Jackson *et al*., 2004). These observations were in agreement with those reported by Singh and Donaldson, 2000. However, checked wood did not always follow this pattern. In 28 % of checking cells lignin was lowest in the S_1 layer (Figure. 4.5 C and D), although the checked wood did not have a significantly lower concentration of lignin in the S_1 layer based on the greyscale analysis (Jackson *et al*., 2004).

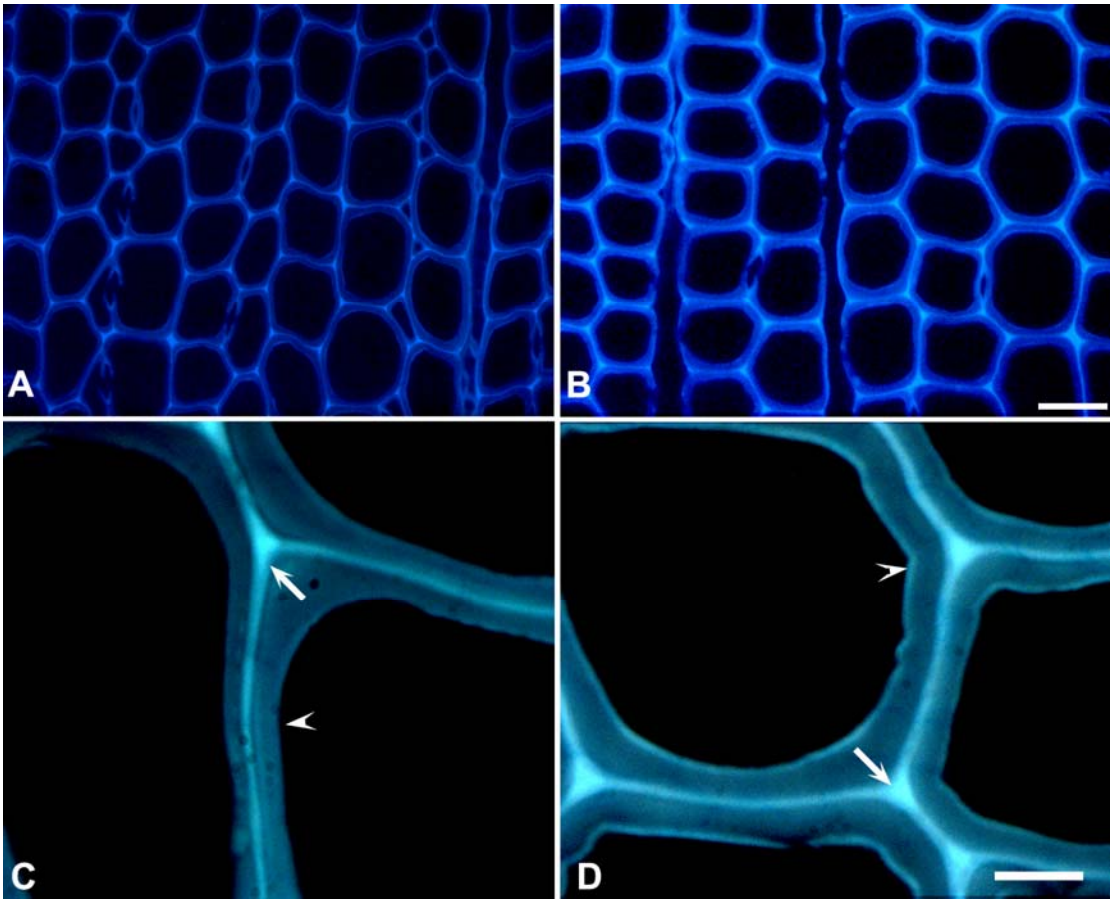


Figure. 4.4 *UV autofluorescence of lignin in checked (A, C) and non-checked wood (B, D). Lignin was high in the compound middle lamella of non-checking wood (indicated by arrows in B and D) compared to checked wood. The S_3 layer also displayed more lignin in the non-checked wood (indicated by arrowheads) than in the checked wood.*

The histochemical light microscopy, epiflourescence microscopy and TEM study indicated the association of low lignin with checking. These observations were in agreement with earlier observations (Singh & Donaldson, 2002; Donaldson, 2002) where it was seen that there was less lignin in the compound middle lamella and secondary cell wall of checked wood. The lower levels of lignin could be detrimental to the quality of wood creating weak zones that could lead to checking.

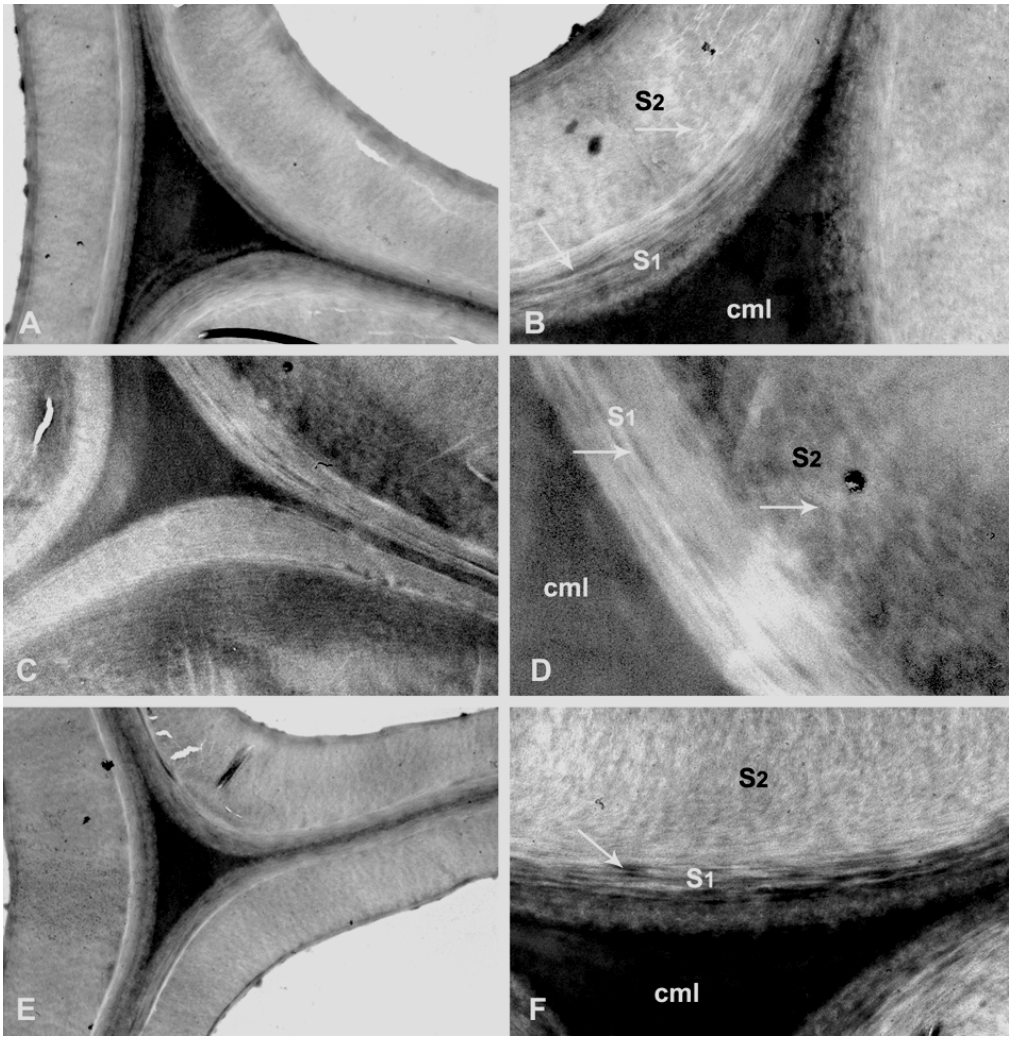


Figure 4.5*: Lignin staining was lower in the compound middle lamella of checked wood. Sections were stained with potassium permanganate to detect the lignin in the cell wall layers and were observed with Transmission Electron Microscopy. A, C and D were from severely checked samples. B was from a moderately checked sample. E and F were from non-checked wood. Lignin was always most concentrated in the compound middle lamella, typically followed by the S_1 and S_3 layers, with the S_2 layer having the least amount of staining the lowest staining (A, B, E and F). In some of the severely checked wood; however, the S_1 wall layer contained the least lignin (B, C). Arrows indicate regions of uneven lignin distribution in the S_1 and S_2 wall layers. Image courtesy T. Putoczki.*

The observations of the micrographs made it clear that the compound middle lamella had lower levels of lignin in the checked wood. Lignin was also low in the S_2 and S_3 wall layer of the checked wood in comparison to the non-checked wood.

The checked and non-checked wood was further subjected to biochemical lignin analysis to determine lignin content. The lignin content of the earlywood portion of growth ring seven in each of the discs was determined by Klason lignin and acetyl bromide assays (Klason lignin content was determined by Veritec, SCION, Rotorua, New Zealand and the acetyl bromide lignin content was calculated by T. Putoczki, Putoczki, 2006). Both the biochemical tests showed that the total lignin content in the checked and non-checked wood was not statistically different from each other. However, the values obtained do not represent of the distribution of lignin in the cell walls, nor do they provide the composition of the lignin in the cell walls (Putoczki, 2006).

Sample group	Acid insoluble lignin[a]	Acid soluble lignin[a]	Total lignin[a]	ABL
Severe	28.6 ± 1.02	0.70 ± 0.02	29.3 ± 1.0	22.9 ± 1.11
Moderate	28.7 ± 0.66	0.72 ± 0.04	29.4 ± 0.68	22.4 ± 0.79
Low	29 ± 0.19	0.74 ± 0.02	29.7 ± 0.17	27.6 ± 2.24
None	29.1 ± 0.78	0.68 ± 0.03	29.8 ± 0.80	23.70 ± 1.66
[a] Values determined by the Klason method.				

Table. 4.1 *Klason and acetyl bromide lignin content of ground wood material from severe, moderate, low and non-checking radiata pine oven-dried discs. The Klason method determines acid insoluble and acid soluble lignin from which the total lignin content is calculated. Values represent percent lignin (w/w). Data are ± standard error of the mean of two samples (Putoczki, 2006)*

This study was part of collaborative project between T. Putoczki and myself. T. Putozcki carried out the biochemical assays and the TEM study. (Jackson *et al.*, 2004)

Therefore, it seems that it is the distribution and the extent of the individual cell wall lignification, and the tracheid dimensions, that could contribute more towards wood strength and resistance to wood flaws like checking.

Some earlier studies a show similar relationship between low lignin levels in the cell wall and checking (Donaldson, 2002; Gindl, 2001). As discussed earlier, some of the fracture studies (Donaldson, 1995, 1996; 1997) showed that the cell wall fractured in regions of reduced lignification. Lai and Iwamida (1993) showed that on delignification the wood fractured at the compound middle lamella. Hence, decrease in the lignification of cell wall layers could lead to decrease in the strength of the cell wall (Donaldson, 1995). Weaker cell walls may not be able to withstand the forces that are generated during drying leading to splitting of cell walls and formation of checks. Donaldson (2002) has reported something similar in the tracheids of drought stressed pines, where the tracheids with little or no secondary wall lignification collapsed as a result of water tension and affected the solid wood properties. The lignin in the wall contributes to the strength of the cell wall (Wardrop, 1981; Whetten *et al.*, 1998; Gindl, 2002; Gindl *et al*., 2002; Gindl & Teischinger, 2002). A decrease in lignin levels could thus adversely affect the cell wall making it weaker and susceptible to stresses that can lead to the development of wood defects like checking.

A close correlation exists between lignin content and cell wall morphology such as cell dimensions and cell wall thickness (Larson, 1994). There is an increase in the cell wall thickness that occurs due to the lignification of the cell wall layers (Boyd, 1973). The results of our earlier study (chapter three, section 3.3.6) found that the checked wood had thinner radial walls, and the current microscopy observations indicate that checked wood has lower levels of lignin in the cell wall layers. This combination of low lignin content and thin radial cell walls seems to lower the compressive strength of the cell wall making it vulnerable and prone to checking. The checked wood had thin walled tracheids and the lower lignin content in the cell walls further aggravated the conditions, hence when the wood was subjected to drying it could not withstand the drying stresses and developed checks.

Lignification progresses from outer region of the wall towards the lumen, and the amount and the chemical characteristics of lignin vary across the wall and among cell

types (Donaldson, 2001). Lignin binds the cell wall into a rigid composite capable of supporting the weight of the tree and resisting the tension of the xylem sap as it ascends to the leaves (Donaldson, 1994). Lignin is deposited within a complex carbohydrate matrix, which varies in composition from one cell wall region to another (Donaldson, 1994, and references therein). It forms a featureless structure in the middle lamella forming chemical bonds with hemicellulose and pectin compounds to form a single unified material, while in the secondary wall lignin occupies the spaces between cellulosic microfibrils to form a lamellate structure (Donaldson, 1994). A variation in the distribution of the lignin in the cell wall layers was observed and this could be due the variation that already exists in the carbohydrate matrix. It is thus likely, that the checked wood had disturbances in tracheid formation right from the initial stages of its development that continued into the later stages of tracheid differentiation like lignification.

The mechanical behaviour of the cell walls is influenced by the more ductile lignin- hemicellulose matrix (Bergander & Salmen 2000) that is temperature sensitive (Hepworth *et al*., 1998). Generally drying of wood by thermal treatment is accompanied by cleaving of lignin-polysaccharide complex as by organic acids released from hemicelluloses that lead to changes in the physio-chemical properties of wood composites, or sometimes lead to the formation of secondary lignin-carbohydrate linkages (Košiková *et al*., 1999). These changes that take place in wood due to heating could also play a role in weakening of the wood leading to the development of checks in the wood.

When the cell wall layers are subjected to, drying it has been demonstrated that substantial stresses develop in the individual secondary cell wall layers (Thuvander *et al*., 2001). The stressed cell wall layers will move and rotate slightly with respect to each other due to the presence of the cellulose microfibrils that lie at an angle to the longitudinal axis. The strongly lignified compound middle lamella region can act like a rubber and dissipitate energy when the cells wall layers and the cells move with respect to one another. According to Booker and Sell (1998), the middle lamella region is like a rubber and consists of three dimensionally connected chain molecules that can move with respect to each other when shear stress is applied. In other words, the compound middle

lamella has the function of energy absorber at the cellular level. Lignin maybe considered as a binder between the individual cells (Gindl *et al*., 2002). If there is a decrease in lignin content, in the compound middle lamella region, there will be decrease in the efficiency of the layer to absorb stresses and this could lead to the shearing and delamination of the cell walls and development of checks. The compound middle lamella thus can resist delamination of the double cell walls and prevent checking by an energy absorption mechanism (Booker& Sell, 1998).

Lignification has a distinct effect on cell wall architecture (Gindl *et al*., 2002). The unlignified cell wall consists of cellulose microfibrils organised as cellulose-hemicellulose strands embedded in a hemicellulose sheath (Gindl *et al*., 2002). During lignification, the spaces between the cellulose and the hemicellulose strands, and possibly part of the hemicellulose sheath covering the cellulose, are filled with lignin (Hafrén *et al*., 1999). The spaces that exist in the unlignified wall are filled with water that is held there by molecular and polymolecular forces bonding (Stamm & Smith, 1969). During lignification, these are the spaces that could be filled with lignin (Wardrop, 1981). The incorporation of lignin in the cell wall layers results in lowering of the cell wall moisture (Boyd, 1972). The lowering of the moisture content is desirable as there will be less anistropic shrinkage (section 3.3.3) in wood during drying and less likelihood of checks developing in wood. The hydrophilic cellulose and hemicellulose are mechanically weak when wet and hence the hydrophobisation of the cell wall material by lignin not only protects the cellulose and hemicellulose content but also adds to wall strength (Dinwoodie, 1975; Gindl, 2001). Hence, the improvement of the mechanical properties of the cell wall could be attributed to the hardness and stiffness of the matrix material of the impregnated cell wall (Müller *et al*., 2003). The higher concentration of lignin contributes both indirectly through increase of packaging density and directly through measured higher hardness in mature cell walls to the strength of the cell walls of wood (Gindl *et al*., 2002). The lower the lignin content of the cell wall material (like in checked wood), the lower the strength of the wall, and the more vulnerable the walls will be to stresses and likely to form checks.

The matrix substances tend to shrink isotropically, while the cellulose microfibrils form a rigid framework and do not shrink as much during drying processes

(Yamamoto *et al*., 2001). The cellulose microfibrils shrink less in the length direction but more in the transverse. However, the longitudinal shrinkage increases with increase in microfibril angle (Barber & Meylan, 1964). Thus, it can be seen that the shrinkage and elastic anisotropy in the conifers can also be accounted for by the distribution and the nature of the cell wall substances (Boutelje, 1962), the proportion of the thick walled fibres in the tissue, the thickness of S_2 relative to S_1, and variations in lignification of the cell walls layers in the wood (Boyd, 1977). The lignified cell walls shrink less than the unlignified ones (Wardrop, 1981). Checks are known to form in wood due to high levels of differential shrinkage in local regions, and it is likely that they will be initiated in stress concentration zones of poorly lignified vessels and ray cell wall contact (Mackay, 1972; Ilic 1999a; b). If the lignin content is low in the cell walls the compression strength of the wood including the capability of the cell walls to resist collapse during drying is lowered and could lead to collapse of cell (Singh & Donaldson , 2000). One of the possible explanations for this is that when wood is heated to high temperatures the lignin in the wood is softened above its glass transition and there are changes in the chemical structure of lignin. These changes in lignin and its lower content in the cell wall can make the wood more susceptible to checking (Singh & Donaldson, 2000). Hence, during the process of drying when the wood is subjected to the differential shrinkage stresses the lignin hemicellulose matrix is important for the cell walls to resist the negative pressures developing within the cells. The checked wood had lower levels of lignin in the cell wall making it more vulnerable to the stresses leading to the formation of checks. In a comparative study between hardwood and softwood, it was found that there was more shrinkage in hardwood than in softwood. One of reasons for this shrinkage was attributed to the low lignin content of hardwood (Schroeder, 1972). Necesany (1966) was of the opinion that lignin did play a role in the shrinkage of cell walls and that middle lamella was an important factor influencing both the swelling and shrinkage of cells. Lignin in the cell walls could have a tendency towards restraining effect against dimensional change (Schroeder, 1972).

The S_3 wall layer had low lignin levels in the checked wood as compared to the non-checked wood. The S_3 wall layer and the warty layer are usually considered less important as they are thinner than the other secondary cell wall layers (Terziev & Daniel,

2002). However, Booker and Sell (1998), state that the S_3 wall layer plays a very important role in strengthening the cell against collapse caused by water tension. The warty layer and the S_3 layer are of vital importance as they can influence the transport processes during drying. The collapse and checking of the cell wall is due to the contact of these layers with the tree sap, which is evacuated during drying (Terziev & Daniel, 2002). The low lignin levels in this layer could affect its strength and leave it exposed to water tension forces and this could be one of the factors leading to checks.

Lignin content not only affects the thickness of the cell wall, moisture content but also strength of the cell wall. The lignin in the cml as well as in the secondary wall is very important for the integrity of the cell wall when subjected to drying stresses. It can help to withstand theses differential forces, keeping the cells together and resist checking.

4.3.3 No changes were observed in with respect to pectin distribution

The compound middle lamella (Figure. 4.6) stained the most with ruthenium red, which indicates the pectin was mainly located in this cell wall layer. There were lower concentrations (faint pink stains) of pectins observed in the secondary wall layers. Ishii *et al.*, (2001) have reported similar distributions.

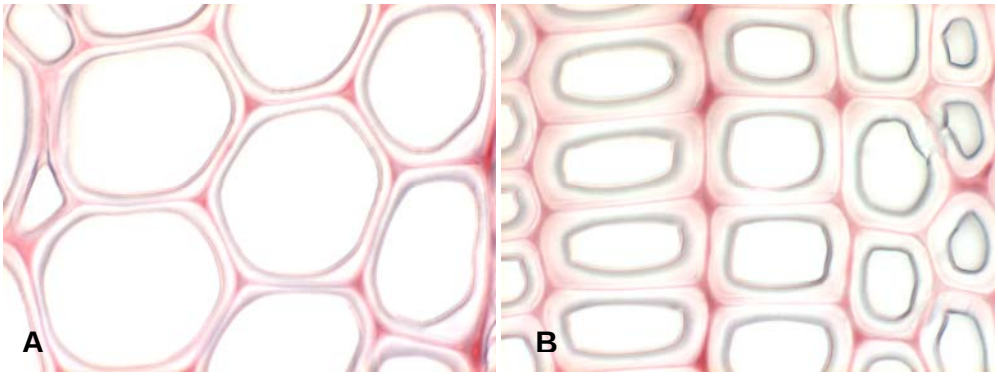


Figure 4.6*: Ruthenium red staining showed that pectin was found primarily in the cml. Pectin distribution did not vary with intra-ring checking in earlywood. In latewood, there was a thicker middle lamella in checked wood samples. Light microscopy done at 100x magnification.*

A TEM study was also carried out to see if we could see any differences in the concentration and distribution of pectin in the individual cell wall layers. As seen in the electron micrographs, no significant differences were detected in the pectin level or distribution in either the checked or the non-checked wood (Figure. 4.7). Pectin staining was strongest in the compound middle lamella for both the checking and non-checking samples (Figure. 4.6 and 4.7). Such a distribution of pectin is expected, as the pectins are mostly concentrated in the middle lamella and primary cell walls (Willats *et al.*, 2001).

The unlignified middle and the primary cell wall layers in both softwood and hardwood consist of pectic substances (Hafrén *et al*., 2000). Immunolabelling has shown the presence of the pectin in the middle lamella/primary walls in both cambium and mature wood (Guglielmino *et al.*, 1997).

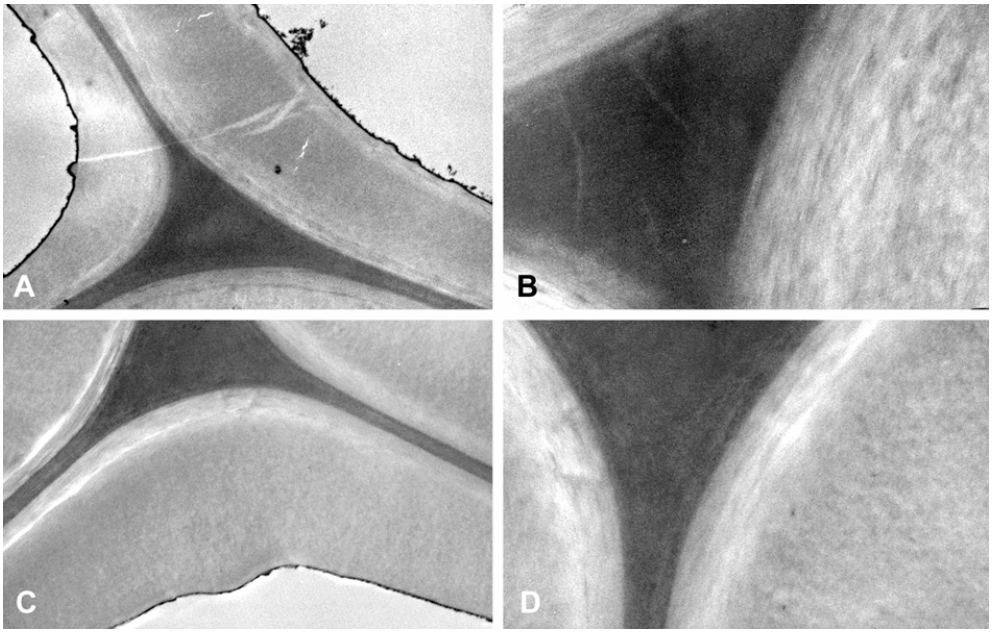


Figure. 4.7 *Pectin concentration was highest in the compound middle lamella of the checked and non-checked wood. The electron micrographs shown in the figure are transverse sections of radiata pine subjected to methylamine lignin extraction followed by the hydroxylamine ferric chloride reaction and observed by TEM. The checked wood are represented by the A and B while C and D the non-checked wood. Image courtesy T. Putoczki.*

According to Wimmer and Lucas (1997), pectin plays an important role during lignification of the wood cells. Pectin is a good chelator of Ca^{2+} , and it degrades or is removed prior to lignification of the cell wall, releasing the Ca^{2+} that are used during the process of lignification. The positively charged Ca^{2+} bind to the negatively charged lignin groups and this in turn could affect the lignification of the compound middle lamella region and its mechanical properties.

When the different stages of differentiation were analysed using immunolabelling it was found that the middle lamella/ primary wall of the differentiating xylem cells were rich in acidic pectins (Guglielmino *et al.*, 1997). According to Tereshima and Fukushima (1988), the acidic pectins may lead to low pH during the process of compound middle lamella lignification, leading to a more condensed lignin structure in this cell wall layer. The deposition of lignin in this porous pectin-rich environment of the compound middle lamella probably leads to a different structure of lignin in the compound middle lamella layers compared to lignin deposited in the secondary wall layers (Hafrén *et al*., 2000).

Some of the current research also indicates that pectin may play a role in stabilizing and strengthening the cell wall through interactions with other molecules (Ryden *et al.*, 2003; Hu and Brown, 1994; Tibbits *et al.*, 1998; Kobayashi *et al.*, 1999; Guglielmino *et al.*, 1997). The arabinose and galactose units act as links between hemicelluloses and lignin (Adler, 1977). As such, one would assume that changes in pectin would correlate with changes in wall extensibility, which in turn can cause changes in cell shape during growth such as the large tracheids associated with checking.

4.4 Summary

During the process of drying, the wood is subjected to various drying stresses that impact on the tracheids, whose ability to resist these stresses is in turn influenced by their dimensions and chemical nature. According to its properties, the wood will either withstand the stresses or give in to the stresses and develop defects such as checking. Lignin is one of the most important chemical constituent of the cell walls, as it imparts strength and rigidity. The impregnation of the cell wall with lignin decreases its bound water content thus lowering its moisture content and increases its thickness adding to the strength of the cell wall. The lowering of the moisture content and increase in cell wall

thickness are desirable properties as they help the wood cells to resist shrinkage and collapse (section 3.3.2 and 3.3.3) as well as checking during drying.

Homogenous lignification across the wood leads to stable wood structure that is less likely to develop intra- ring checks. It is perhaps not the total amount of lignin in the cell wall that is important but the distribution and extent of lignification of individual cell wall layers that is more critical for imparting strength. Lignin composition varies not only from plant to plant, but it also varies across individual cell wall layers (Hepworth *et al*., 1998). Most crucial is the lignification of the compound middle lamella region. From observations of the checks (section 2.3.2), it is clear that the splits occur predominantly at the compound middle lamella and S_1. It is in the S_1 layer that Maurer and Fengel (1991) observed a distinct change from lignin-rich to lignin-poor lamellae and this could favour check formation. Though lignin plays an important role in resistance to collapse and shrinkage other anatomical features like tracheid length, shape and diameter change rapidly with distance from the pith towards the bark and their effect on wood, properties also need to be looked into (Saranpaa *et al*., 1998).

Though pectin differences were not observed in the checked and non-checked wood, it does seem to play a role in lignification and cross-linking thus influencing the integrity of the cell wall and possibly helping in the additional strengthening of the cell wall layers to resist the stress forces. Multiple interconnections between microfibrils greatly increase their buckling resistance (Booker& Sell, 1998). After looking at the tracheids that make-up the major bulk of the radiata pine wood in chapters two, three and four the next chapter explores the other anatomical features of the wood like ray, resin canals, and pits and their involvement in checking of radiata wood.

Chapter Five

Structural flaws may determine initiation site of check

5.1 Introduction

Checking is a seasoning flaw that develops during drying and mostly occurs in the earlywood zone of the growth ring. One of the contributing factors for the development of the checks in wood is the wood structure (Cown *et al.*, 2003). As discussed in section 1.4, softwood has a simple and homogenous structure, mainly composed of tracheids. In radiata pine wood the interlocking pattern of the tracheids form the majority of the wood structure. If this composition was constant throughout it would form a highly stable homogenous structure. However, the uniformity of this pattern is disrupted by the presence of rays and resin canals. These areas have been put forth as sites where potential flaws like checking can develop in wood (Jackson & Nair, 2003). This chapter describes the study that was conducted with an aim to determine the anatomical structures of radiata wood and their relationship with the wood checking.

5.2 Material and Methods

5.2.1 Experimental Design

Thirteen half discs cut from the 60 mm oven-dried discs of radiata pine that were provided by WQI Ltd, New Zealand were used for the study. The discs without any check were categorised as non- checked discs. The discs with checks were categorized

into severe and moderate checked discs depending on the varying number of checks in the growth rings and the number of growth rings affected (Figure. 2.1). The observations were restricted to the growth ring seven to minimize the variations that occur across, and between growth rings.

5.2.2 Sample preparation for scanning electron microscopy

From each checked disc, five checks were collected for the study. The checks were excised from growth ring seven of the main disc with hammer and chisel. A part of the check for investigation was selected and, the surface trimmed with a sharp razor blade. The pieces of checks were mounted on stubs (ProSciTech, Australia) and sputter coated (Polaron E 5000, USA) with gold palladium coating for 2 minutes at 20 miliampheres to give a coating that is approximately 100°A thick. The checks were observed wit a help of Leica 440 scanning electron microscope at varying magnifications ranging from 65 to 15,000x.

5.2.3 Image analysis

The exposed ray area was marked in Adobe Photoshop (version 6). The surface area occupied by the rays was determined using Image Pro Plus (version 4.5 for Windows, Media Cybernetics Inc, USA). Similar steps were carried out to study the area occupied by resin canals in the wood.

5.2.4 Digital photography

Digital photographs of the transverse surface of the checks were obtained using a Nikon D1x camera fitted with a Nikon 60mm Macro lens. The arrangement of the resin canals was studied using these photographs.

5.3 Results and Discussion

5.3.1 Wood rays could be one of the sites for check initiation

The observation of the SEM images showed that 10% of the checks had rays associated with them. A similar sort of relationship between rays and intra-ring checking has been reported in some earlier studies (Donaldson, 1995; Wang & Young, 1996). In six cases of the total 60 cases observed, the check seemed to follow a ray (Figure. 5.2). Miller and Simpson, (1992) made a similar observation where they reported, that the plane of the check was quite likely to follow the fusiform rays or resin canals. In some of the cases observed (Figure. 5.1 C, D, E and F), the ray was seen sometimes attached on one side of the check. It seemed that the ray separated from the adjacent tracheid at the compound middle lamella. As reported by Wang and Young (1996), during drying of wood, failures develop within the ray walls, and between ray wall and the adjacent tracheids, this could contribute in check development. One of the possible reasons for this to happen could be the nature of the ray cell wall. In their observational study, Singh and Donaldson (2000) pointed out that the ray parenchyma cells were unlignified and showed evidence of collapse, while the ray tracheids showed more lignification than adjacent axial tracheids and resisted collapse induced by drying. As the rays are mostly unlignified they have low compression strength and greater susceptibility to moisture effects (Pang, 2002), hence, they are more likely to collapse on drying. Moreover, the rays can also influence shrinkage in radiata pine wood, which can lead to possible development of checking. Pang (2002) stated that the rays in radiata pine influence shrinkage in two ways, by increasing rigidity in the radial direction, and by permitting collapse in the tangential direction, due to wide spacing, and the thin nature of the tangential cell wall of the ray cells. The rays contribute to a higher tangential shrinkage and lower radial shrinkage (Pang, 2002) that could influence the development of check in wood. However, the ray may not be the causal factor, as not all checks showed the presence of a ray. Each tracheid comes in contacts with at least one ray (Esau, 1967), yet this point of contact between ray and tracheid does not always develop a check, suggesting that additional factors could be involved.

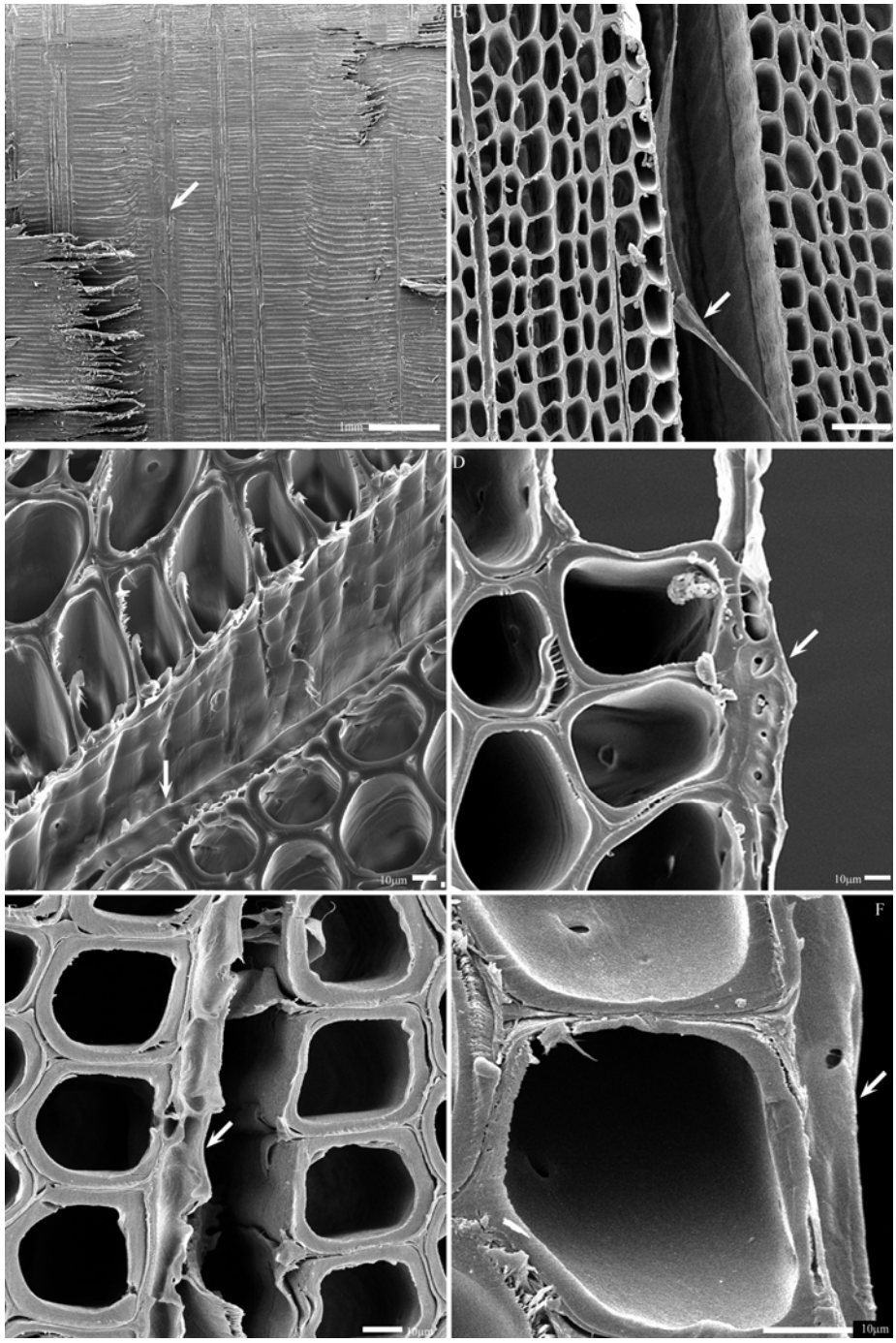


Figure. 5.1 *Scanning electron micrographs that display the association of rays with the checked surface of radiata wood. The radial surface of the check exposed a number of rays on its surface. The arrow indicates a ray (A). In some cases, a part of the ray tracheid was seen to be attached to one side of the check and torn away from the adjacent tracheid, most likely at the compound middle lamella (B, C, D, E and F).*

5.3.1.1 Rays occupied more radial surface in checked wood

Image analysis was conducted on the scanning electron micrographs to further explore the relationship between rays and check. The main aim of the study was to analyse the proportion of the checked surface area occupied by ray tissue. For this comparative study, of checked surface to a non–checked wood surface, a piece of non-checked wood sample was split open along the radial dimension and observed with the SEM microscope. The checked and the non-checked surfaces were then analysed to see the surface area occupied by rays (Figure. 4.2).

Severely checked samples had an average of 19.97 $\pm$ 6.23 % of their surface area occupied by ray tissue (Figure. 5.2A), while moderately checked samples had 15.27 $\pm$ 7.32% (Figure. 5.2B) and the non-checked samples had 8.57 $\pm$ 2.52(Figure. 5.2C). The proportion of ray tissue did vary with checking (ANOVA P = 0.04434151, Appendix 2). Therefore, it seems there maybe a relationship between the surface area occupied by the rays and the likelihood of development of checks in wood. Hence, this analysis suggests it that a proportion of ray tissue makes wood could be susceptible to checking. These findings are support of an earlier study by Donaldson (1996), who found tentative evidence for the relationship between higher frequencies of ray (ray/mm) to be associated with checked wood.

5.3.2 Resin canals may not be involved with initiation of check

Some of the checks observed with the help of scanning electron microscope were seen to be associated with resin canals. However this was seen only in 10% of the checks observed (n=60 checks). In the severely checked samples, two checks had resin canals associated with a check, and four checks with resin canals were observed in the moderately checked samples. The resin canals in the check were located anywhere from the midpoint of the check to the termination of the check in the latewood. The thin-walled parenchyma cells surrounding the resin canal in some of the cases observed had collapsed (Figure. 5.3 C and D). The epithelial cells of the resin canals in the secondary xylem of stem except in *Pinus* have thick lignified cell walls (Wu & Hu, 1997).

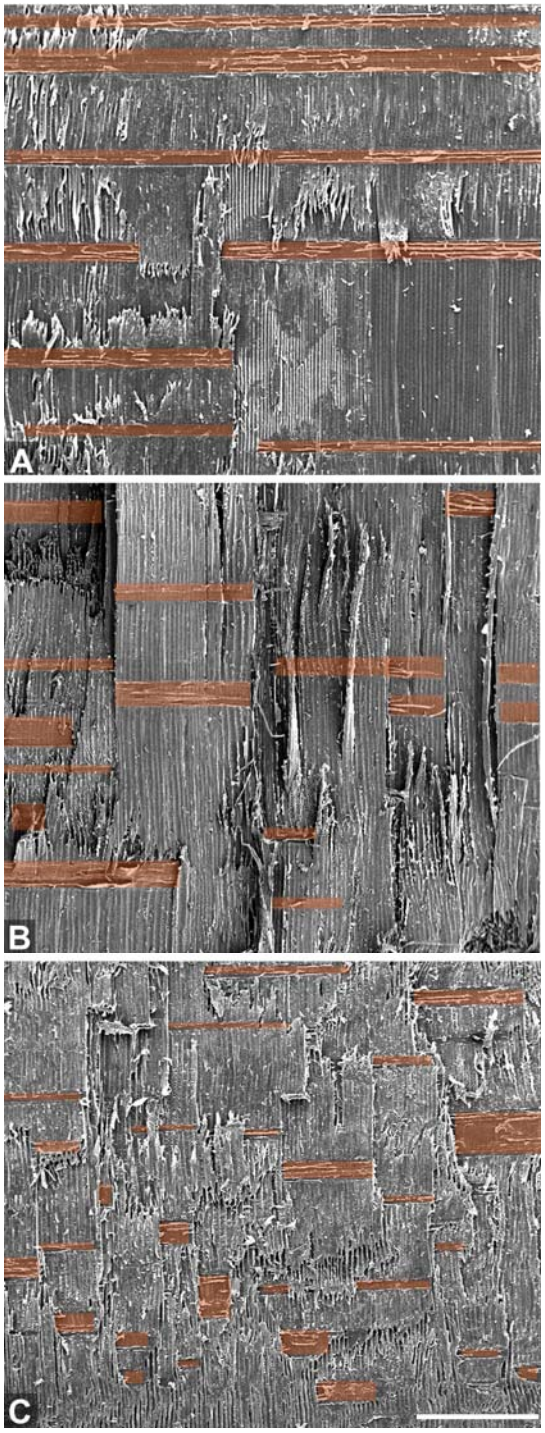


Figure. 5.2 *Ray tissue occupied more of the radial surface of the check in severely checked wood (A) compared to moderately checked wood (B). Non-checked wood split open along the radial surface had even less surface area occupied by ray tissue (C). Scale bar=1mm*

There is likelihood that the thin epithelial cells of resin canals in radiata pine could be a point of weakness in wood. However, the number of resin canals that were seen in association with checks was very low. Hence, usually the resin canals are not the point of initiation of checks.

5.3.2.1 Resin canals had a scattered distribution in checked wood.

Similar to the rays further analysis was carried out on the number of resin canals that were present per unit area in the checked wood (ANOVA, P= 0.332). No significant difference was observed between checked and non-checked wood. However, the pattern of distribution of resin canals varied. The resin canals were scattered from the middle of the earlywood up to the latewood in all of the severely checked samples (Figure. 5.4A), the same was observed for moderately checked wood, whereas, in the non-checked wood the resin canals were arranged in a linear manner (Figure. 5.4B). The results of the observation are summarised in Table. 5.1. The severely checked wood displayed only scattered arrangement of resin canals, while 60% of the moderately checked samples showed scattered arrangement. In contrast, only 25% of the normal wood showed the scattered distribution.

The normal resin canals in pines are evenly distributed (Panshin & deZeeuw, 1970) as opposed to the traumatic resin canals that appear sporadically in wood (Panshin & deZeeuw, 1970; Jane, 1970). The majority of the resin canals observed both in checked and non-checked wood seemed normal and not traumatic as, even when the distribution of resin canals was scattered, their occurrence was not sporadic, and their size was normal, according to Panshin and deZeeuw (1970) the traumatic resin canal are larger than the normal resin canals. The non-checked wood had most of its resin canals in the earlywood-latewood transition region in accordance with the usual occurrence of resin canals in *Pines* (Panshin & deZeeuw, 1970). As per the literature review by Wimmer and Grabner (1997), the resin canals are concentrated in the earlywood-latewood transition or the latewood region. However, in the checked wood some resin canals were seen in the earlywood region.

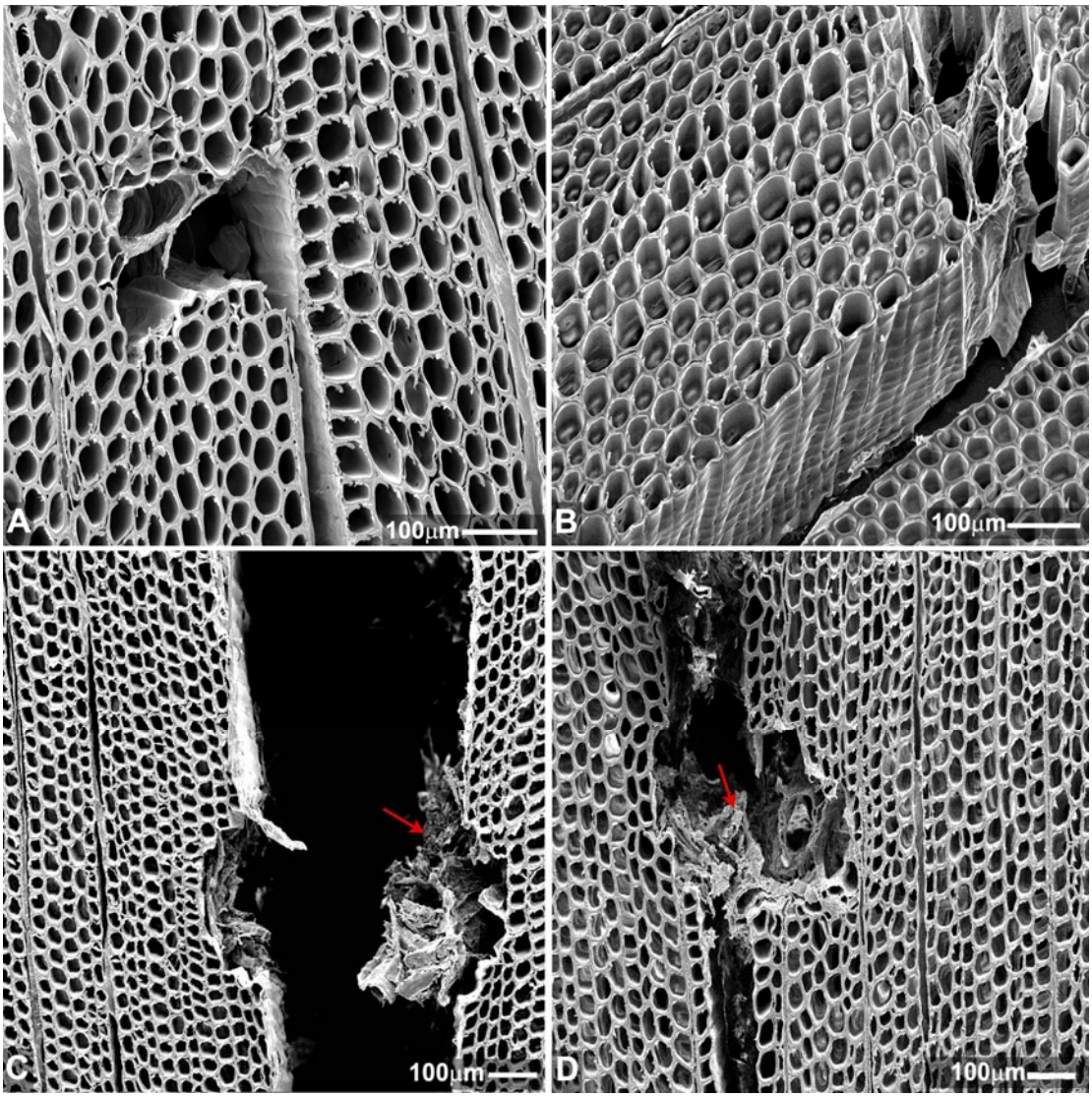


Figure. 5.3 *Scanning electron micrographs showing the transverse face of checks from severely (B) and moderately (A, C, D) checked samples. The check passed through the resin canal ripping the thin-walled epithelial cells (arrow in C). The epithelial cells appear to have collapsed (arrow in D). Collapsed tracheids can be seen along the check in B and C.*

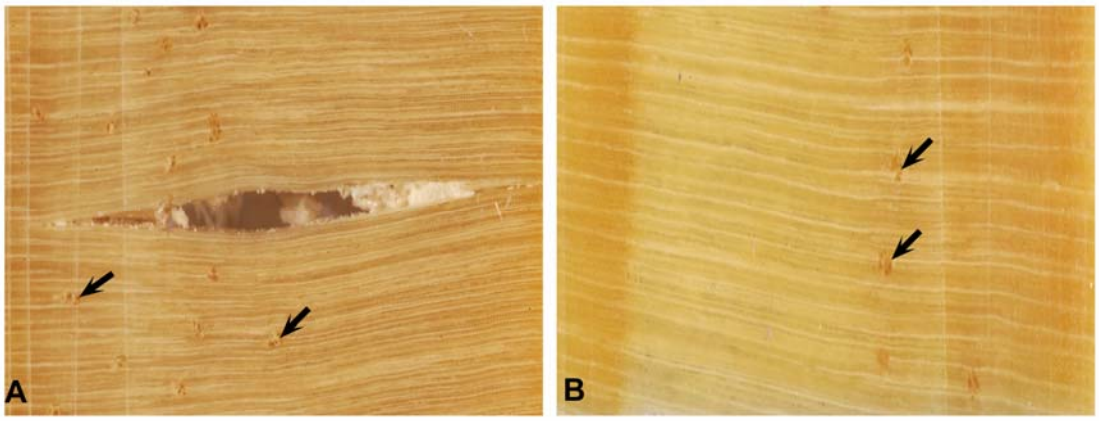


Figure. 5.4 *Photograph of the transverse face of wood from a severely checked sample (A) and a non-checked sample (B). In the checked sample (A), the resin canals are scattered in the radial dimension (arrows). In the non-checked sample (B), the resin canals have a nearly linear arrangement.*

There is no direct association between resin canals and checking in wood. However, it is possible that the resin canal arrangement could be an indicator of some developmental disturbances during the process of wood formation.

Resin canal formation in wood is linked to phytohormones. Some of the experiments have shown the involvement of auxin and ethylene in resin canal development. Both auxin and ethylene can stimulate resin canal development (Fahn & Zamski, 1970; Yamamoto & Kozlowski, 1987). Some researchers have also pointed out that, many of the effects previously considered to be induced by auxin are possibly the result of auxin induced ethylene formation (Imaseki *et al*., 1982; Abeles *et al*., 1992). Although there is criticism that most of these studies of resin canals and phytohormones involved shallow wounding during application of hormones, and wounding in itself can induce traumatic resin canal formation (Franceschi *et al.*, 2002). Jasmonates are another group of endogenous phytohormones that is known to induce traumatic resin canals (Franceshi *et al*., 2002; Hudgins *et al*., 2004). Franceschi *et al*., (2002) induced traumatic resin canal formation by applying jasmonates to the bark of *Picea abies*, without wounding.

Sample number	Type of wood	Resin canal linear	
1	Severe	No	100%scattered
2	Severe	No	
3	Severe	No	
4	Moderate	No	60% scattered
5	Moderate	No	
6	Moderate	Yes	
7	Moderate	Yes	
9	Moderate	No	
10	Non-checked	Yes	25% scattered
11	Non-checked	No	
12	Non-checked	Yes	
13	Non-checked	Yes	

Table. 5.1 *Resin canal distribution was scattered in checked wood, whereas only 25% of the non-checked wood displayed scattered resin canal arrangement.*

They suggested that it is unlikely for jasmonate to directly induce traumatic resin canal development, possibly the jasmonate induces changes in the ethylene or auxin production that in turn results in the activation of traumatic resin canal formation.

A number of different biotic and abiotic factors have been known to cause traumatic resin canal formation in conifers including insect and fungal attack, wounding, frost and other physical trauma (Franceshi *et al.*, 2002). Once the plant perceives stress, a number of stress responses get activated, and some of these involve interactions of hormones with each other (Morgan & Drew, 1997). Hormones like ethylene and jasmonates that respond to stress also influence auxin. There is evidence that some steps of the ethylene signal transduction pathway can be influenced by stress (Morgan& Drew, 1997), and ethylene can affect the basipetal flow of auxin (Lev-Yadun & Aloni, 1995).

The basipetal polar movement of auxin in turn controls cambial activity and xylogenesis (Uggla *et al*., 1998). Some jasmonate induced processes reduced auxin levels, while some auxin stimulated physiological processes inhibited jasmonates (Saniewski *et al*., 2002).

The process of xylogenesis is regulated by hormones (Savidge, 1996), and any change in a hormone will affect tracheid differentiation. From the above discussion, it can be seen that ethylene, jasmonates and auxin can influence one another and the process of wood formation. Therefore, when there is an increase in the number of resin canals and the early development of resin canals in checked wood, there is a possibility that this could be due to hormonal imbalance. Further research could also be carried out to see if the earlier development of resin canals does give any clue about the wood quality of radiata pine.

5.3.3 Larger pits were associated with checking

Pits are areas in the tracheids that serve as passages for the sap to pass from one cell to another (section 1.4.2.2). A study was conducted to see if there were any differences between the pits of the checked wood and the non-checked wood. The radial surface of checked and non-checked wood was observed with the help of scanning electron microscope. The earlywood along the radial surface exposes the pits well and is also the area that gets mostly affected by checking. Hence, the area was selected for the study. Image analysis of the electron micrographs helped us to measure the pit apertures (Figure. 5.5 arrowhead) and pit borders (Figure. 5.5 smaller double-headed arrows) of the pits seen in checked and non-checked wood. It was found that severely checked wood contained average pit apertures of 4.71$\pm$ 1.15 μm, while they were 5.58$\pm$1.16 μm in moderately checked wood and 3.80$\pm$0.97 μm in non-checked wood. Hence, checked wood tends to have larger pit apertures (ANOVA P=0.01070309, Appendix 2).

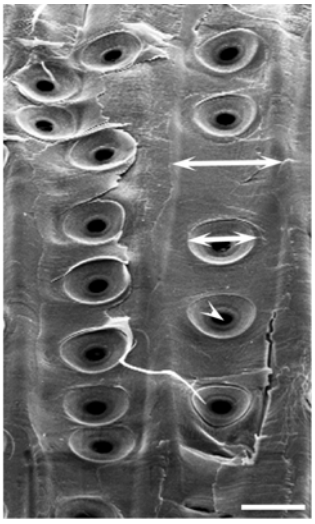


Figure. 5.5 *Pits were observed along the radial face of the check. The pit aperture (arrowhead), pit border (small double-headed arrow) and tracheid (large double-headed arrow) widths were all measured. Scale bar=20μm*

Similarly, the pit border was also larger in checked samples, severe samples being 14.44 ± 2.30 μm, moderate being 17.08 ± 3.09 μm, and non-checking being 11.43 ± 2.53 μm (ANOVA P=0.003841311, Appendix 2). The measurements were made on oven-dried discs, hence, there is a possibility of overestimation while measuring. This has been rightly pointed out by Thomas& Nicholas (1966). However, the trend of checked wood having larger pit apertures and pit borders is still quite evident.

Jane (1970) while discussing the development of checks or cracks in the tracheids during drying identified the pits as being a point of weakness in the wall. The mechanical properties of the mature earlywood tracheids are more heterogenous than the latewood due to the presence of numerous pits in earlywood (Mott *et al*., 2002). This could also be the case in the checked wood where the pits could be exerting their influence and playing a role in check formation. As seen from the data analysis the checked wood had bigger pit apertures and pit borders as compared to the non-checked wood. One of the possible explanations for this is that bigger pits could lead to faster drying of wood, as water is able to escape from wood more easily during drying. The faster drying of wood especially at higher temperatures can lead to development of checks (Simpson *et al*.,

2002). In addition the pits can also influence radial tracheid shrinkage during drying (Pang, 2002), which could also play a role in formation of checks.

When water is lost from wood during kiln drying, aspiration of pits usually occurs (Meylan & Butterfield, 1972; section 1.4.2.2). The aspirations of pits could lead to the development of differential water tension in wood that is able to cause checking. Booker (2004) suggested that during drying water molecules diffuse through the cell walls and leave sapwood cells causing suction in the water contained in the cells. The earlywood cell walls are thinner with higher water content; whereas the latewood cells have thicker cell walls, and many of the cells are not water filled, (they are full of gas bubbles Kininmonth & Whitehouse, 1991). All these as per Booker can cause differential contraction between earlywood and latewood, which can lead to the development of checks in wood. From this, it seems highly likely that aspiration of pits could play an important role in differential contraction between earlywood and latewood that can possibly lead to checking.

The amount of adhesion force between water and pit membrane necessary to effect pit aspiration depends on the membrane structure (Liese & Bauch, 1967). The latewood pits are less prone to aspiration than earlywood (Butterfield & Meylan, 1982), as they have a denser margo than earlywood pit membranes (Thomas, 1967), lower pit membrane diameter (Thomas & Kringstad, 1971) and a thicker torus (Liese & Bauch, 1967). The earlywood pit membranes in sapwood are rarely incrusted, in comparison to the heavily incrusted latewood membranes from both sapwood and heartwood (Thomas, 1969). These characteristics that are seen in the latewood pits cause an increase in the force required for closure, so that even the high surface tension of water does not produce aspiration in all pits (Liese & Bauch, 1967), leading to lower frequency of latewood pit aspiration. If the pit aspiration frequency is high, the differential water tension in the wood will be high and the wood will be more prone to develop checking during drying.

In the sapwood, the pits are open while they are closed in the heartwood (Fengel, 1970). The pits of ray tracheids in heartwood show complete incrustation of pit membranes, the pits between ray tracheids and between ray and longitudinal tracheids are closed not by aspiration but by sealing the membranes (Fengel, 1970). Bauch and Berndt (1973) investigated about 100 species of coniferous wood for the chemical composition

of pit membranes particularly aromatic substances. They found that in quite a number of species pit membranes in sapwood had phenolic substances, while in heartwood lignification of pit membrane takes place besides the development of other polyphenols. It is likely that the lignified pit membranes and sealing of the pits prevents the heartwood from being affected by differential water tension during drying and hence the heartwood does not develop checks. It is possible that with further research in the development of pit membranes and the process of pit aspiration during drying in radiata pine, there could be more control gained over the process of drying, to minimize differential water tension in wood and prevent checking.

5.4 Summary

Structural features that disrupt the uniformity of the interlocking pattern of the tracheids such as ray, resin canals could be the areas of weakest link in the radiata pine wood that could cause checking. From the study that was carried out it seems that in checked wood there was more surface area of the wood occupied ray tissue. It is likely that rays could play a role in checking. Few resin canals were associated with check. However, the pattern of arrangement of resin canal could possibly lead to an indication about a woods tendency to check. It was seen that checked wood usually had a more scattered arrangement of resin canals compared to non-checked wood. The checked wood also seemed to have larger pits compared to the non-checked wood.

After the study on the checked and non-checked wood, organ culture trials were carried out to understand the process of xylogenesis and in turn understand some of the factors that could play an important role in influencing the wood quality.

Chapter Six

The role of auxin in xylogenesis of radiata pine

6.1 Introduction

The findings from the study of checking in radiata pine showed that the checked wood varied not only in the cellular dimensions but also had differences at the cell wall chemical composition level (chapter three and four). The various stages of wood formation and the interplay of the nutrients and hormones that influence the wood formation were examined, in order, to understand the differences that were found in the study of checked wood. Organ culture was a tool that allowed such an investigation. Researchers have used different methods to study xylogenesis but it seemed that organ culture clearly had an advantage over some of the methods already in use. This method to culture wood *in vitro* not only helped overcome the complexities that might arise due to intact plant responses but is also a system that is closest to what happens in an intact tree during xylogenesis (Savidge, 1993).

Radiata pine is a plantation species of high economic value for the forest industry in New Zealand. The tree was introduced in New Zealand as it is versatile and has a high growth rate. The growth rate of radiata pine exceeds $20m^3$/hectare/year (McCurdy & Keey, 1999). The high growth rate of radiata pine could be an indicator that the tree has high auxin levels. Auxin is the main phytohormone that controls many developmental programmes in plants by influencing cell growth, cell division and cell specialization (Callis, 2005). It is highly likely that these auxin levels influence the process of wood formation in the tree. The study presented in this chapter was performed to understand the role of auxin in wood formation in radiata pine.

6.2 Material and Methods

6.2.1 Experimental Design

The organ culture method used by Savidge (1993) and Leitch (1999) was found best suited for the study of xylogenesis in radiata pine, although some modifications were carried out to adopt it to the radiata pine. The organ cultures were prepared from radiata pine trees and grown on defined media where the auxin concentrations were varied. After a growth period of 30 to 60 days, the organ cultures were collected and subjected to various analyses to investigate the efficacy of the organ cultures and to study the affect of auxin concentrations on xylogenesis of radiata pine wood.

6.2.2 Tree material

Radiata pine trees growing on two sites were used for the study. Two healthy looking trees from the Rotorua site of SCION, New Zealand, were cut and de-branched. The trees were wrapped in bubble wrap to minimise damage during transportation and flown to Christchurch, New Zealand, packed in an insulated container with ice packs. The stem segments were stored at 4°C until organ cultures were made. The segments of the main stem axis at breast height were used to make the organ cultures and the rest of the tree was discarded. One tree from the University of Canterbury site at Burnham, New Zealand, was also used for the study. The tree was cut and de-branched in the similar manner as above and stored at 4°C. The cultures were made from segments within a week (although it was observed that the trees stayed viable for 30 to 40 days after they had been cut and stored at 4°C).

6.2.3 Culture media and its composition

The media used for growing the organ cultures contained all the typical ingredients of plant tissue culture media (White, 1967; Barnett & Asante, 2000). The composition used for the current study was first reported by Allen *et al*. (1988), modified by Savidge (1993) and further modified to make it conducive for the growth of radiata pine organ cultures.

The details of the composition of the culture medium are listed in Appendix 4. When preparing a litre of media some of the components like sucrose (2% w/v, 20,000 mg/L), agar (0.8% w/v. Difco Bacto-agar, 8,000 mg/L, BD Biosciences, Sparks, USA) and myo-inositol (25 mg/L) were weighed separately and directly added to the medium. Other components of lower concentrations were made up as stock solutions and added in appropriate amounts (Appendix 4). 1-naphthalene acetic acid (NAA, Sigma, USA) was added to the medium as per the required concentration. The concentrations used were 0.003 mM, 0.03 mM, 0.3 mM and 3 mM. The lower concentrations of auxin represented by 0.003mM, 0.03 mM and higher concentrations represented by 0.3mM and 3 mM NAA concentrations. No other phytohormones were added to the medium. The pH of the medium was adjusted to 5.8 with 0.1 M KOH. The medium was then autoclaved (121°C and 140 kPa, for 20 minutes). The sterilised medium was poured into sterilised petri-dishes (90 mm diameter, BioLab Scientific, Auckland, New Zealand) to a depth of 3 to 4 mm, sealed with plastic film (GladWrap®) and stored in dry place at room temperature.

6.2.4 Preparation of organ cultures

The trees that were used to make the organ cultures were approximately 10 years old and felled during the months of March and April. The procedure followed was mainly based on Savidge (1993) and Leitch (1999).

6.2.4.1 Sterilization of tree material

A disc of about 6 cm was cut from the main stem segment. The bark, the outermost surface of the disc was scrubbed with detergent (Pyroneg, Global Science) and then rinsed thoroughly with running tap water. The disc surface was then scrubbed with dilute bleach (approximately 2%, Domestos®). Using a sharp razor knife the outer rhytidome was pared from the entire surface of the disc, and care was taken that all the resin cysts were removed from the surface. If the resin cysts remained it was found that there was an increase in the tendency for the organ cultures to develop fungal contamination. This observation was made in the current study, as well as by Savidge (1993). This process exposed the underlying phloem layers. According to Savidge (1993) and Leitch (1999), it was crucial to maintain a thickness of 1 to 2 mm of crushed and

non-crushed phloem layers to maintain an active cambium. Reducing the thickness of the phloem layers could lead to either killing or heat shocking the cambium during the later stages of flame sterilization, rendering it inactive and the experiment futile. For the current study, a phloem layer of 1 to 2mm was maintained. The disc thus prepared was rinsed with 95% ethanol and placed in a specially designed holder in a laminar flow cabinet (Figure. 6.1).

6.2.4.2 Flame sterilization

The disc placed on the specially designed holder was sprayed with 95% ethanol. The surface of the disc was ignited and permitted to burn until the flame died. The process was repeated if the colour of the phloem remained whitish; it was flamed until the colour of the phloem turned a light orange-red. During the flame sterilization, process the disc was rotated slowly through 180° with the help of the specially designed holder (Figure. 6.1).

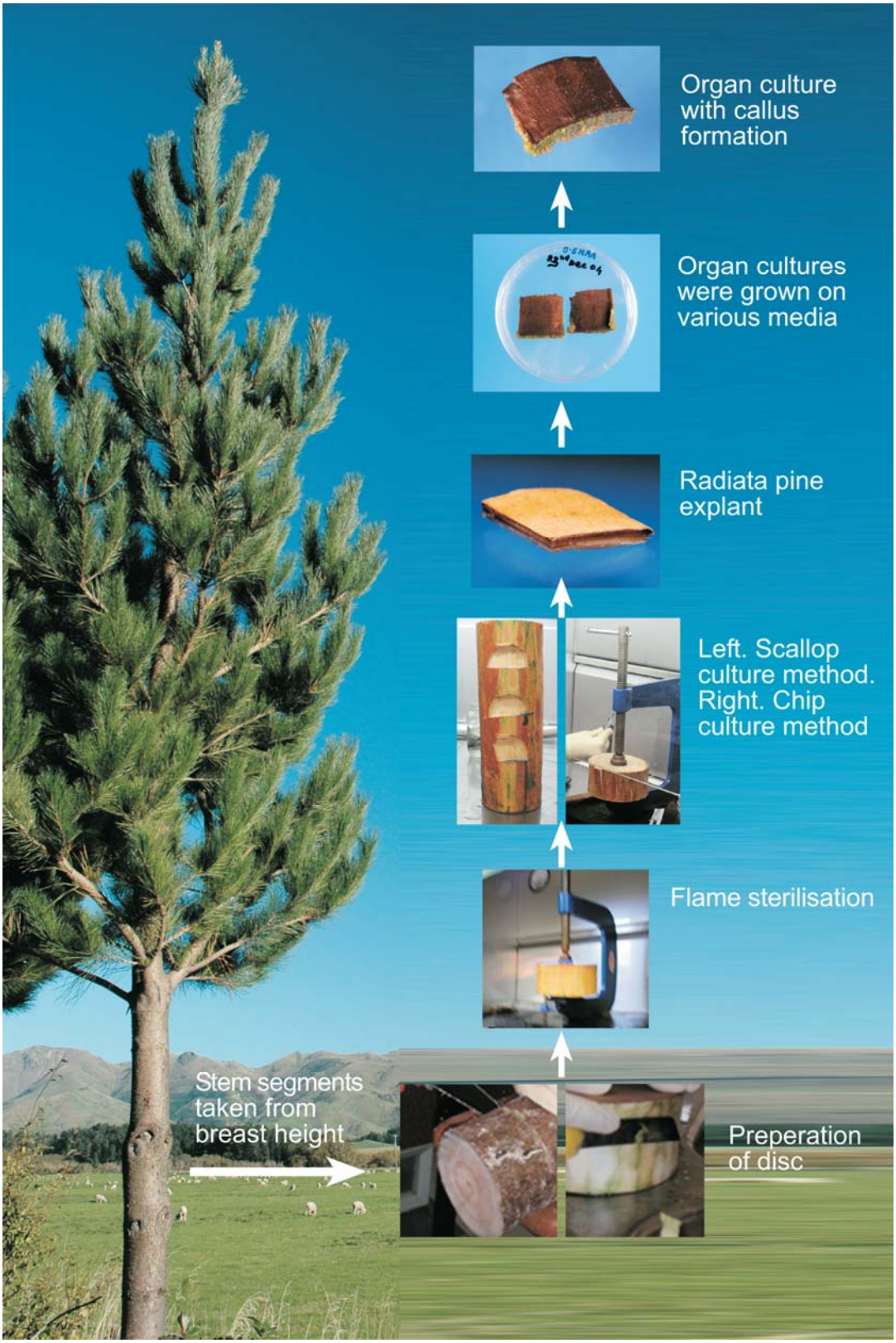
6.2.4.3 Excision of explants from sterilised disc

Two techniques were used for the excision of the explants from the sterilised disc. The 'chip explant method was same as described by Savidge (1993) and the 'scallop explant method' similar to that of Leitch (1999).

6.2.3.4.3.1 Chip explant method

For this method of excision, the protocol described by Savidge (1993) was followed. Using a flame sterilised hammer, chisel and a sharp knife pieces of approximately 2 to 3 cm axial x 1 to 2 cm tangential x 1 to 0.5 cm radial were excised from the disc (Figure 6.1). These explants were then placed on the sterile growth medium with varying concentrations of auxin. Two explants were placed in each petri-dish in such a way that the xylem layer that was exposed by excision was in direct contact with the medium. The petri-dish was then sealed with plastic film (GladWarp®) and placed in growth room. One of the explants was stored in 4% paraformaldehyde in ¼ x PBS as control.

Figure. 6.1 Preparation of the organ cultures. *Disc was cut from the main stem segment using a mechanical saw. The bark, the outermost surface of the disc was scrubbed with detergent and rinsed thoroughly with running tap water, then scrubbed with dilute bleach. Using a sharp razor knife the outer rhytidome was pared from the entire surface of the disc and all the resin cysts were removed from the surface. The disc placed on the specially designed holder was sprayed with 95% ethanol, the surface ignited and permitted to burn until the flame died. The explants were then excised from the disc using two techniques the 'chip explant' and the 'scallop explant' method. The explants of the radiata pine were cut under sterile conditions. They were placed on petri-dish containing growth medium with varying auxin concentrations. (Photos of disc preparation, flame sterilization and excision courtesy, T. Putoczki)*


Organ culture with callus formation
Organ cultures were grown on various media
Radiata pine explant
Left. Scallop culture method. Right. Chip culture method
Flame sterilisation
Preperation of disc
Stem segments taken from breast height

6.2.4.3.2 Scallop explant method

The method for scallop explant preparation was the similar to the one used by Leitch (1999). This method is different from the chip explant method in two aspects. The first is that a bigger piece of disc approximately 12 to 15 cm was cut initially from the main stem segment and secondly the final excision from the flame sterilised disc is different from the chip explant method that is being described here further.

A flame sterilised hammer, chisel and a sharp knife were used to excise pieces of explant approximately 2 to 2.5 cm axial x 3 to 4 cm tangential x 1 to 0.5 cm radial from the flame sterilised disc. A cut was made at an angle, and perpendicular to the long axis of the disc, and then a semi-circular shaped explant was cut out (Figure. 6.1). Similar to the chip explant the scallop explants were placed on the sterile medium with the exposed xylem layer in contact with the medium. The scallop explants were, different in shape they also did not include the cells that were exposed along the edge of the disc. As the initially cut discs bigger, more scallop explants could be cut along the flame sterilised disc (Figure 6.1).

6.2.4.4 Marking of the initiation of culture growth

In organ culture studies conducted by Savidge (1993), the cambium was dormant, hence the latewood boundary was used as a marker for the start of the growth in culture. Leitch (1999) used formation of axial parenchyma in organ cultures as a marker. Axial parenchyma formation takes place as a response of the cambium when detached from the main stem and grown in culture conditions (Savidge, 1993; Leitch, 1999). The same markers could not be used for the current study as the formation of the axial parenchyma was not observed and the radiata pine cambium in New Zealand does not attain total dormancy (Harris, 1991).

In order to mark the initiation of activity in organ cultures for the initial experiments sterile needles were used to pierce the cambium prior to the excision of the explants from the sterilised disc. A similar procedure referred to as ‘the pinning method’ has been used earlier to mark point of wood formation (Nobuchi *et al.*, 1995).

The piercing of the cambium provided a reference point in the organ cultures so that it was easier to demarcate the location of the original wood and the wood grown in culture. The layers of wood above the piercing were new layers of cells formed under culture conditions and the layers below it were formed earlier in the tree. This method was discontinued due to increase in contamination.

Another method that was tried to mark the culture growth was that of injecting 0.1% Janus green dye in PBS with sterile needles into the cambial region of the explant prior to excision from the sterile disc. This was done to mark the status of layers of wood in explants at the time of their preparation. It was hoped that the dye would stain the cambial cells and would be retained during their subsequent development. However, it was observed that injecting dye mostly damaged the cambial cells and the dye did not spread. Furthermore, this method also led to an increased incidence of contaminations and was therefore abandoned during the last year of the study. The preferred method for monitoring growth in the culture system was the cell counting method. The number of cells in the control explant was compared to the number of cells of organ cultures and the difference between the two was used as a confirmation for the culture growth.

6.2.4.5 Culture growth conditions

The growth conditions for the organ cultures grown by Savidge (1993) and Leitch (1999) were different to the current study with respect to temperature and light conditions. The organ cultures were grown by Savidge (1993) and Leitch (1999) at 25 and 24°C respectively under continuous light conditions. In the current study, the organ cultures were grown at 21°C with 23.5 hours of light per day.

6.2.5 Growth period of the organ cultures

Savidge (1993) grew *Larix laricina* cultures for five weeks and reported as many as 20 new tracheids per radial file, based on this finding the organ cultures of radiata pine were allowed to grow for a period of 4 to 24 weeks and then collected and subjected to further analysis.

6.2.6 Fixation of organ cultures post culture growth

After a period of growth usually between four to six weeks, the organ cultures were collected and fixed. The fixative used was 4% formaldehyde in ¼ PBS.

6.2.6.1 Preparation of the fixative

Preparation of 16 % formaldehyde

The 4% formaldehyde was prepared from 16% formaldehyde. 8 g of paraformaldehyde was dissolved in 40mL of distilled water and heated to 60°C in fumehood with constant stirring. Once the temperature of the solution was achieved, the heating was switched off and 1 M NaOH was added drop by drop until the solution was clear. The volume was then adjusted to 50mL and filtered.

Preparation of PBS

8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 were dissolved in 800mL of distilled water. The pH was adjusted to 7.4 and finally the volume was adjusted to 1 L.

Preparation of 4% formaldehyde in ¼ x PBS

To make the fixative, 25 mL of the 16% formaldehyde and 25mL of the 1x PBS were mixed and the final volume was adjusted to 100mL using distilled water.

6.2.7 Sample collection for analysis

Smaller pieces were cut from the organ cultures that have been stored in the fixative and used for further analysis. The sample collection was done only from the middle region of the organ cultures to avoid any edge effect in the samples collected.

6.2.8 Preparation of permanent slides

The samples collected were processed according to the protocol described in section 3.2.2.1. Wax sections were cut and stained with safranin-fast green and toludine blue stain to carry on with further analysis and histochemical studies. The same method

as described in section 3.2.2.1 was followed for the preparation of the permanent slides from the current samples.

6.2.9 Histochemical analysis

Two stains were used to study the wood anatomy and visualise the lignified tissues of the organ cultures, safranin- fast green stain and toluidine blue. Safranin-fast green staining procedure that was followed was the same as described in section 3.2.2.2.

6.2.9.1 Toluidine blue staining

The wax sections of the organ cultures were first dewaxed and then stained with toluidine blue, following the protocol described below. The lignified tissues (such as the mature tracheids of xylem layers) when stained with toluidine blue displayed a greenish blue colour (Donaldson *et al*., 2001), while the unlignified tissue (like phloem, cambial and RE regions) displayed a bluish to violet colour.

For dewaxing, the slides were taken through a xylol series (100% xylol for 1 minute followed by two 5 minute changes). The slides were then taken through rehydration series of different grades of ethanol 50: 50, 100 % xylol: 100% ethanol; 100%, 95%, 80% and 70 % ethanol, all for one minute each. This was followed by two rinses of distilled water lasting one minute each. The slides were then transferred to 0.05% aqueous (w/v) toluidine blue stain for one minute. The slides were then thoroughly rinsed in water until the excess stain had been washed off. The stained slides were then dehydrated again by taking them through an ethanol series (95%ethanol two changes lasting one minute each followed by two stages of 100% ethanol of one minute duration each) and then xylol (one change of 50: 50 (v/v) 100% xylol: 100% ethanol of 30 seconds; followed by 3 changes of 100% xylol for one minute and five minutes duration each). The sections were mounted in DPX and cover slipped (BioLab Scientific).

6.2.10 Light microscopy

Light microscopy was carried out on the safranin-fast green and toluidine blue stained slides with the help of Zeiss microscope that was connected to a Zeiss Axioshop

digital camera for collecting images. The images were used to visualise lignin and do image analysis.

6.2.11 Epifluorescence microscopy

The same procedure was followed as described in section 4.2.4.2 (chapter four) was used to make observations of lignin distribution in the cell wall layers of organ cultures

The dewaxed sections mounted in distilled water were observed using Olympus lX 70 inverted microscope attached to mercury lamp. Digital images were collected using a Cool Snap CCD camera (RS Photometrics) using standardised exposure conditions.

6.2.12 Preparation of samples for TEM microscopy

Small pieces of approximately 2 mm radial x 2 mm longitudinal x 3 mm tangential were cut from the organ cultures. These were then fixed and embedded in Spurr resin. The embedded samples were sectioned, stained with uranyl acetate and Sato's lead and observed with a Hitachi TEM microscope at 75 kV or Phillips TEM at 80 kV.

6.2.13 Sample fixation and embedding for preparing sections for TEM

Samples were washed with 0.1 M cacodylate buffer (Electon Microscopy Sciences, Fort Washington, USA. pH 7.2, twice with each wash lasting five minutes each). The samples were then fixed in 4% glutaraldehyde (Electon Microscopy Sciences, Fort Washington, USA) in 0.1 M cacodylate buffer under vacuum for 3 hours. The samples were then washed in 0.1 M cacodylate buffer (thrice with each wash lasting 10 minutes each) and then post-fixed in 1% osmium tetraoxide (Electon Microscopy Sciences, Fort Washington, USA) for 3 hours. The samples were again washed in 0.1 M cacodylate buffer for 10 minutes dehydrated with graded ethanol series (20, 40, 60, 80, 90 (v/v) and 100% ethanol each stage lasting for 30 minutes). The dehydrated samples were put into 100% acetone twice (each stage of 30 minutes duration). The samples were then infiltrated with Spurr Resin (Electon Microscopy Sciences, Fort Washington, USA, low viscosity medium hardness kit). The first step of resin infiltration was with 25%

Spurr Resin and 75% acetone and the second step was 75% Spurr Resin and 25% acetone. The samples were left in the first stage overnight at room temperature with slight agitation, while they were left in the second stage for approximately 6 hours at room temperature. The samples were then embedded in 100% Spurr resin using embedding capsules and cured at 70°C overnight. Ultrathin section (approximately 100 nm) were cut with LKB2128 Ultrotome (Uppsala, Sweden) using a diamond knife (Micro Star, Huntsville, USA) and placed on formvar coated 100 mesh copper grids (Electon Microscopy Sciences, Fort Washington, USA) ready for the staining protocol. Fixation, dehydration and resin infiltration were performed in fumehood.

Preparation of formvar

1.75% (w/v) formvar was prepared in chloroform. Glass slides were coated with this solution, and the formvar film was floated off onto water. Copper grids were then placed on the film, lifted out on a piece cardboard and left to air dry.

6.2.14 Staining of ultra-thin sections for TEM study of lignin

The sections on the grid were ready for the staining protocols as described in the section below.

6.2.14.1 Uranyl acetate and Sato's lead

Ultra-thin sections on copper grids were submerged in 1% uranyl acetate (BDH, Poole, England) in 50% (v/v) ethanol in distilled water) for 10 minutes. The grids were then rinsed in 50% ethanol twice with each rinse lasting five seconds and two changes of distilled water. The samples were then submerged in Sato's lead for five minutes and rinsed in distilled water twice for five seconds each. These grids were then placed on filter paper and air dried.

6.2.14.2 Potassium permanganate staining

Ultra-thin sections on copper grids were submerged in freshly prepared 2% (w/v) potassium permanganate (BDH, Poole, England) for one hour (stains lignin Singh & Daniel, 2001). The grids were then washed with distilled water for five seconds and

submerged in 0.5% (w/v) citric acid (BDH, Poole, England) for 30 seconds. The grids were then rinsed with distilled water thrice each rinse lasting five seconds and then left on filter paper to air dry.

Preparation of 2% (w/v) potassium permanganate

250 mL of distilled water was boiled to reduce to 100 mL. 2 g of potassium permanganate crystals were added while the water was still hot. The solution was then allowed to cool and was ready to use once cool.

6.2.15 Development and scanning of the TEM negatives and TEM image collection

The stained copper grids were observed with the help of Hitachi H-600 TEM at 75kV or a Phillips TEM at 80 kV. The images from Hitachi H-600 were collected on Kodak Electron Image Film SO- 163. The negatives were developed for four minutes in undiluted Kodak D- 19 developer at 20°C. The negatives were then scanned using Epson Perfection 1200U scanner. The images were converted to TIF files and stored on a compact disc. Digital images were collected with the Phillips TEM.

6.2.16 Preparation of samples for the field emission scanning microscopy (FESEM)

Small pieces of approximately 5 to 6 mm radial x 3 to 4 mm longitudinal x 2 to 3 mm tangential were cut from the main fixed organ cultured wood. Samples were carefully trimmed using double edged razor blades, rinsed and treated with 0.5% sodium hypochlorite for 10minutes to remove cytoplasm (the wood becomes colorless) (Abe *et al*., 1995). It also delignifies the wood (removes lignin, though some polysaccharides could also dissolved Chaffey, 2002a). After rinsing in distilled water, samples were then processed through a graded series of amyl acetate and critical point dried. Samples were coated (20 to 40 seconds). Prior to observing with JEOL 6700F field emission scanning electron microscope (FESEM)

6.2.17 Analysis to confirm growth in cultured wood by cell count method

Images of the sections of the organ cultures were collected with the help of light microscopy. Images of the light microscopy of an entire set (that is organ cultures of same tree, same disc cultured on same day) including the control for that set were printed. Usually images collected at 20x magnification stained with toluidine blue were used for this procedure. Sometimes if the print was not clear then the digital images were used. Cells were counted in the four major regions of the organ cultures. Four to six radial files were counted for and averaged. The three regions that were counted were the 'cambial region', the 'RE region', and 'the developing cells region' (Figure. 1.3). The first region is the 'cambial region' this was identified by the thin walled small cells, which were either undergoing active cell division or were the newly divided cells. The second area 'the RE region' followed the cambial region and comprised of cells that were thin walled and undergoing radial expansion. The cells of this region were recognised by thin walls and larger radial dimension. The 'developing' region consisted of cells undergoing lignification. The cells of these regions were counted right upto the point where the first mature tracheid was encountered. The mature tracheid was identified by thicker secondary cell walls and higher lignification of the secondary cell wall layers.

6.2.18 Image analysis for lumen area, radial length and tangential length

The images collected at 20x magnification were used for the image analysis of tracheid lumen area, radial length and tangential length of the organ cultures. The software was calibrated for 20x magnification and images were analysed. The cells along the edges or incomplete cells were deleted with the toggle option of the software. The average of the measurements was obtained using Microsoft Excel. The data was then statistically analysed using Statistix version 8 statistical software programme (Analytical software, Tallahassee, USA).

However, the same procedure could not be used for the cambial cells. The measure tool of the software was used for this. Lines were drawn in the radial direction and

tangential direction and the measured data was transferred to the Micrsoft Excel sheet. The measurements of the lengths were then used to calculate the area of the lumen of the cambial cells (length x breath or radial length x tangential width).

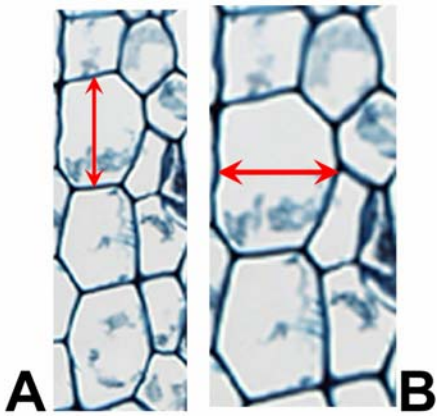


Figure. 6.4 *Transverse sections of cultured wood stained with toluidine blue were observed and their radial length and tangential width were measured using Image Pro Plus. The arrows represent the direction of radial length (A) and tangential width (B) measured in the cells observed.*

6.2.19 Image anaylsis for cell wall thickness

The images collected at 20x magnification were used for doing the cell wall thickness measurements as well. An area of interest was selected and demarcated from the rest using AOI tool from the image analysis programme (Image Pro Plus). The total area occupied by the selected area was recorded. The area occupied by the cell lumen was then recorded by following the method described in section 6.2.19. The total sum of the area that these cells occupied within the marked area was measured. Both measurements were recorded in Microsoft Excel. The total selected area was subtracted from the total lumen area. The value obtained was the area occupied by the cell wall of the cells in the selected area. The percentage of the cell wall thickness was then calculated and the data was statistically analysed using Statistix version 8 (Analytical software, Tallahassee, USA).

6.2.20 Analysis of the lignin content

The lignin content in the wood of the organ cultures was analysed using Klason lignin method and acetyl bromide assay for lignin. Pyrolysis gas chromatography and mass spectrometry was also used to measure the lignin content of cultured wood. The area of lignin occupied in cml/S_1 that was visualised by epifluorescence microscopy was measured using Image Pro Plus (section 4.2.5, chapter four) and analysed.

6.2.20.1 Preparation of samples for lignin content analysis

The organ cultures growing on different auxin concentrations for a period 16 weeks were collected and subjected to lignin analysis. The phloem layer was removed from the organ cultures. The wood was then dissected to obtain the wood in the region of new growth, and allowed to air dry. The dried samples were then finely ground using a mixer mill (Retsch GmbH and Co., Germany) for seven minutes at 23 Hz or until the wood powder could pass through 250 µm mesh.

6.2.20.2 Klason lignin analysis

One gram of ground sample of each sample to be analysed was sent to Veritec, SCION (Rotorua, New Zealand). The Klason lignin in the samples was determined by M. Dibley and P. Gray. The acid-insoluble lignin in wood was determined by using the TAPPI Standard Methods T 222 om-88, and the acid soluble lignin content in wood was determined using the TAPPI Useful Method UM 250. A single measurement only could be done on each of the samples due to limited resources. The Klason lignin method generally involves treating the sample with sulphuric acid in order to promote carbohydrate hydrolysis, the lignin isolated by this method is referred to as acid-insoluble lignin (Dence, 1992).

6.2.20.3 Pyrolysis gas chromatography and mass spectrometry

The finely ground samples of the wood of the organ cultures were sent to ensis SCION (Rotorua, New Zealand) for analysis of lignin content by Pyrolysis gas chromatography and mass spectrometry (Py-Gc-MS). The analysis on the samples was

carried out by D. Steward and A. Wagner. For this analysis, samples from high (3 mM) and low concentrations (0.003 mM) of the treatments were used from the same set. Duplicate measurements were performed on each sample. Due to limitation of resources, only one set was used for this analyis. The percentage composition of the identified pyrolysis products was determined for the duplicate analysis using Microsoft Excel.

6.2.20.4 Acetyl bromide assay

The procedure followed for the acetyl bromide assay was the same as the one described by Hatfield *et al.* (1999), and modified by Möller (2001). Perchloric acid was not used in the procedure used for this study as the samples were finely ground and hence, the dissolution of the cell wall material was achieved. Approximately 2.5 to 3 mg of ground wood sample was weighed into borosilicate glass tubes. 0.5 mL acetyl bromide in glacial acetic acid (25% v/v) was added to the samples in the tubes and incubated for two hours at 50°C in a water bath. The tubes were then cooled on ice for about five minutes, then 2 mL sodium hydroxide (2 M) and 2.4 mL glacial acetic acid were added, cooled and vortexed. 0.37 mL hydroxylamine (0.3 M) was added and the mixture vortexed again. This solution was then quantitatively transferred to the volumetric flasks to which glacial acetic acid was added and the final volume made to 25 mL. The absorbance of this solution was then observed at 280 nm against glacial acetic acid with the help of Agilent 8453 diode array spectrophotometer (Forest Hills, Australia), using disposable cuvettes.

The lignin composition was calculated using the equation shown below.

$$\textbf{lignin [\%w/w]} = 100 * (A_s - A_b) * V * a^{-1} * W^{-1}$$

where A_s and A_b = absorbance for sample and blank respectively; V = volume of the solution (L); a = extinction coefficient of the lignin standard (g^{-1} cm^{-1}); W = weight cell wall material (g). An extinction coefficient of 20.09 g^{-1} cm^{-1} was used to calculate the lignin content (Dence, 1992; Iiyama & Wallis, 1988). This assay was performed in duplicate for each sample and two sets were used for this assay.

6.3 Results and Discussion

6.3.1 Successful modification of the culture media

When the first trials of the organ cultures were carried out the media composition was the same as the one used by Savidge (1993). However, some of the organ cultures were also grown with sucrose instead of glucose. The sucrose concentration was varied between 2% (w/v) and 0.2% (w/v) compared to the 4% (w/v) glucose of Savidge (1993) medium. It was found that the organ cultures showed better growth at 2 % sucrose and the organ cultures had green callus formation. In comparison, the organ cultures of radiata pine grown in 4% glucose seemed unhealthy as the wood felt all soft to touch. The organ cultures grown in 0.2% showed very little callus growth. Hence, for further experiments the organ cultures were grown at 2% sucrose concentration. Zajaczkowski, (1973) reported optimum growth in isolated stem segments of *Pinus silvestris* at 2% concentration in the media.

The media used for this study was also modified with respect to the auxin concentrations used for the organ cultures. The auxin used for the organ cultures was in the form of 1-naphthalene acetic acid (NAA). In some earlier studies of xylogenesis, auxin in the form of indole acetic acid (IAA) has been used successfully. However for the current study auxin in the form of NAA was preferred as it is stable in heat, air, light or salts present in the culture media compared to IAA (Leitch & Savidge, 1995) and is equally as effective as IAA in eliciting cambial activity (Torrey & Loomis, 1967). It was also the compound used successfully by Savidge (1993) with organ cultures of *Larix laricina* that showed not only not only cambial activity but also complete wood formation when grown with NAA.

According to Savidge (1993) 0.03 mM of NAA was ideal concentration that supported cambial cell activity and wood formation in organ cultures of *Larix larcina*, and similar observations were made about this level of auxin concentrations in other studies (Zajaczkowski, 1973; Leitch & Savidge, 1995). Hence, this concentration was selected as reference concentration of NAA for the current study of organ cultures in radiata pine. In order to study the affect of auxin on xylogenesis the organ cultures were

grown in varying concentrations of auxin, keeping 0.03 mM as the reference point auxin concentrations of 0.003 mM, 0.3 mM and 3 mM NAA were used to study the impact of auxin on the xylogenesis. The auxin concentrations of 0.003 and 0.03 were considered as low auxin conditions and 0.3 mM and 3 mM were considered as high auxin concentrations for the current study result analysis. No other phytohormones were added to the medium.

6.3.2 Microbial infection and evaluation of preparation of the organ cultures

The preliminary organ culture trials followed the procedure described by Savidge (1993). It was found that more than 90% of the organ cultures were contaminated with microbial infection within 4 weeks of culture growth. The microbial contamination was found to be predominantly fungal infection. This observation was contrary to the one reported by Savidge (1993), where more than 90% of *Larix laricina* organ cultures were found to be free of contamination after 5 weeks of culture growth. Growth plates with no organ culture were used as control and these showed no sign of infection. Hence, it was confirmed that the source of infection was from the organ cultures. Most of the fungal infection contaminating the organ cultures was the endophytic fungus identified as *Pestalotiopsis sps* (N. Cummings pers comm.) (Figure. 6.2). Since the major source of contamination of the organ cultures was an endophytic fungus, the sterilization of the explant material of radiata pine posed a challenge. The previous studies suggested that most of the surface contaminants could be removed by the initial washes and by removing outer layers of bark and resin cysts (Savidge, 1993; Leitch, 1999). The washes of the tree material were done more rigorously and the paring of the bark was done so as not to leave resin cysts, along with extra attention to the sterilisation of all the equipment that was used. These changes in practice led to a decrease in the incidence of contamination. However, still nearly 50% of the organ cultures were being lost due to contamination.

The contamination was always observed along the edges of the organ cultures. Hence, another method of explant excision from the flame sterilised disc was tried. The ‘scallop explant’ method used by Leitch (1999) was tried to make the explants. In this

method, the explants could be excised from the middle portion of the sterilised disc, which could lead to a decrease in the contamination of the explants. When a trial was carried out, it was found that only 25% of organ cultures were seen to be contaminated. Hence, the scallop explant method was found to be more effective in making radiata pine organ cultures.

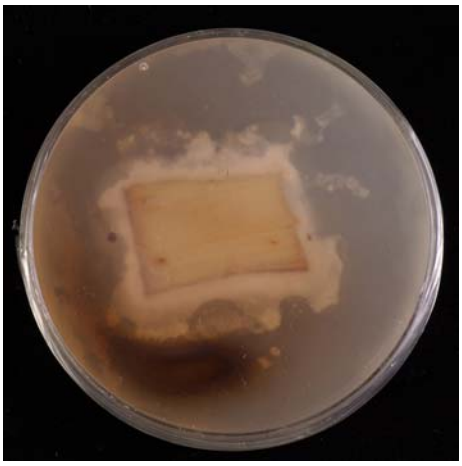


Figure. 6. 2 *The fungal infection contaminating the organ cultures was the mostly due to the endophytic fungus Pestalotiopsis sps.*

6.3.3 Organ culture response to culture conditions in the form of callus formation

Callus develops in the plant as a wounding response (Savidge, 1993). Hence, callus formation was observed along the periphery of the organ cultures as a wounding response to the process of excision during the making of the explants from the sterilised disc. Callus was seen to appear along the periphery of the organ cultures within one week of culture initiation (Figure. 6.3). Leitch (1999) made similar observations where the organ cultures of *E. globulus* showed the presence of peripheral callus within one and a half week of culture growth. Callus formation was taken as an indication that the organ cultures were growing and responding to the treatments. The health of the callus indicated the health of the organ culture, hence the organ cultures that showed the presence of green callus and greater amount of callus content were assumed to have more healthy vigorous growth. However, in the case of the 3 mM auxin concentration the

callus was mostly brownish in colour (Figure. 6.3). When monitored closely there was steady increase in the callus formation along the edges of the organ culture observed during the period of growth, which was taken as indicator of healthy growth of the organ culture. The two studies by Savidge (1993) and Leitch (1999) report a similar kind of relationship between callus and xylogenic responses of the organ cultures. According to Savidge (1993) the 'chips' with green callus have slightly more vigour both in terms of callus growth and number of tracheids produced. However, on further analysis such a relationship between amount of callus and number of cambial cell division and tracheid production could not be clearly established. The callus on the organ cultures was compact in nature and quite similar to the ones reported by the previous studies of Savidge (1993) and Leitch (1999).

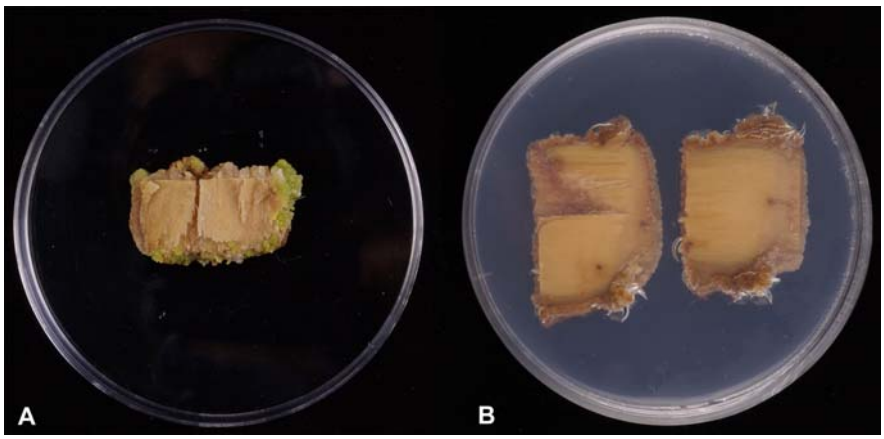


Figure. 6.3 The organ cultures showed green callus formation along the periphery, except in 3 mM auxin concentration there was mostly brownish colour callus that was observed.

6.3.4 Sub-culture and maintenance of the organ cultures

Attempts were made to sub-culture the organ cultures. The organ cultures once sub-cultured did not show the formation of healthy callus, hence, the conclusion that sub-culturing was not a viable option. The organ cultures were transferred after every four weeks into fresh growth medium with a hope to sustain them in an active state for longer. It was observed that despite the supply of fresh nutrients there was no appreciable change. The cultures could not be maintained for long periods by sub-culturing. Most of the callus changed to brown colour usually after a period of 18 weeks growth. This was

taken as an indicator of decline in culture activity. None of the trials were able to revive it. This could indicate that there are other factors that are responsible for growth of wood in cultures that were present in the explants and these are exhausted leading to cultures reaching a point of maximum favourable growth. Similar observations were made by Savidge (1993), who reported that the sub-culturing of the organ cultures was not possible and that cambial activity ceases in organ cultures after a period of time. There could be other growth factors that could lead to growth response of cambium which when exhausted resulted in dormancy of the cambium (Savige, 1993).

6.3.5 Cultured wood displayed colour differences

The cultured wood seemed to have wood characteristics similar to earlywood of radiata pine (section 1.4.1) with radial file continuity of the tracheids when observed under a stereomicroscope. Other organ cultures have shown the development of earlywood as well (Zajaczkowski, 1973; Savidge, 1993). However, there was one obvious difference between the cultured wood and the control and that was the colour of the cultured wood.

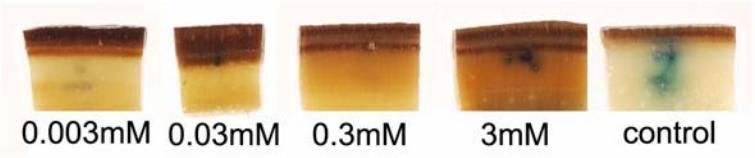


Figure. 6.3 *The cultured wood colour differed from control wood. There was an increase in colour of wood to darker shade of brown with increase in auxin concentration of growth media.*

The wood grown under culture conditions appeared darker with increasing auxin concentration. The wood colour in the control explant was whitish in colour and the cultured wood grown under low auxin concentrations displayed lighter colour wood, although a hint of orange reddish colour was observed. The cultured wood grown in higher concentration conditions shows a much darker colour wood. Overall, the colour of the wood became darker in a dosage dependent manner (Figure. 6.3).

The colour changes were observed consistently in five sets of cultures. Brown colourations of wood due to the production of phenolic substances in response to wounding have been reported earlier (Le Roux & Van Staden, 1991). However, in the current study if the change in wood colour was a wounding response, then it would most likely not be influenced by the auxin concentrations, as the change in wood colour varied, according to the auxin concentration in which the organ culture grew. Another possible reason for this colour change could be due to down regulation of CAD enzyme (Mackay *et al*., 1996). An alteration in the lignification of the cultured tracheids could also lead to a change in the wood colour. A study was conducted to determine lignin levels of cultured wood (described in section 6.3.6). There seemed to be an increase in the hardness of cultured wood. Wood grown on higher auxin concentration (0.3 mM and 3 mM) was considerably more difficult to cut than the control and the wood grown on lower auxin concentrations (that is 0.003mM and 0.03 mM). One of the possible explanations for this change in wood property was attributed to the increase in the lignin content of wood grown on higher auxin concentrations (section 1.7.4, chapter one).

6.3.6 Increase in the number of cell with increase in auxin concentration

Transverse sections of the organ cultures grown in the varying auxin concentrations were observed using light microscopy and the images were then analysed for cell count (described in section 6.2.18). This analysis was conducted on three different sets from two different trees. Usually the cambial cell division increased with increase in the auxin concentration. There was nearly an increase of 70% in the cambial activity with an increase in auxin concentration compared to the control. An increase in the RE cells was also observed with an increase in auxin concentration. The increase in the RE cell number was most in 0.3 mM of auxin concentration. However, in one set the highest auxin concentration of 3 mM did show the lowest number of RE cells. There were approximately 22% more cells within in cultured wood grown on high auxin concentrations (0.3 mM and 3 mM combined) in the organ cultures. There was an increase in the number of cells compared to the control even in the lower auxin concentration though the increase was low compared to high auxin concentration.

In 0.003 mM concentration on an average, there were 10% more cells than in the control. The observations of cell counts in all the regions of the 3 sets has been summarised in the Table 6.2 based on the measurements as shown in Appendix 4. In one set (set 3[c]), there was consistent increase in the number of cells in all three regions of the culture wood observed with increasing auxin concentrations. The variations exist in the behaviour of the organ cultures, though when individually compared to the within set control a pattern of behaviour of the cultured wood appears, where exogenous auxin increased the cellular activity.

Cultures grown in varying auxin concentrations						
	Set 1[a]		**Set 2[b]**		**Set 3[c]**	
Concentration levels mM	0.003,0.03	0.3, 3	0.003,0.03	0.3, 3	0.003,0.03	0.3, 3
Cambial region	High 0.03mM most	High 3mM least	High 0.03mM least	High 3mM most	Low 0.03mM most	High
RE region	Low/High 0.03mM with most	High/Low High with least	High	High 0.3mM with most	Low	High/Low 0.3 mM with most
Developing cell region	High 0.003mM most	No change/ High	High 0.03 mM with most	High 3 mM with least	High 0.03 mM with least	High 0.3mM with most

[a]Rotorua tree, initiated in april cultured for a period of one month.
[b]Rotorua tree, initiated in April cultured for a period of two months.
[c]Burnham tree, initiated in March cultured for a period of one month.

Table. 6.2 *Summary of the cell number observed in cultured wood grown on different auxin concentrations. The increase in the number of cells when observed was highlighted. In set 3 with increase in auxin concentration, an increase in the number of cells is observed in all the 3 regions of the cells observed (Based on measurements in Appendix 4. Statistical analysis in Appendix 4).*

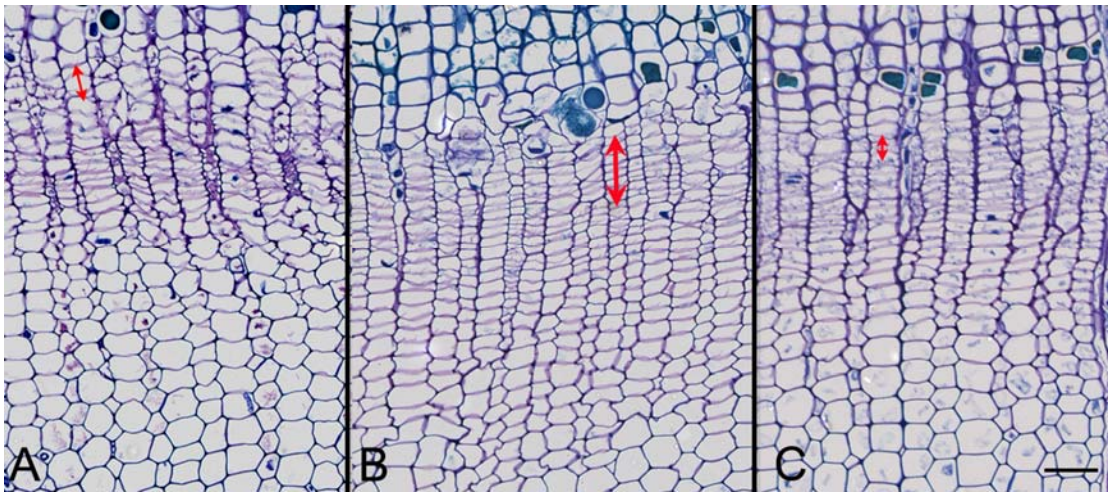


Figure. 6.4 *Transverse sections of cultured wood grown on low and high auxin concentrations stained with toluidine blue were observed. The cultured wood in low auxin concentration (A) had more number of cambial cells than control (C) but lower than the cultured wood grown on high auxin concentration(B). Scale bar =50µm.*

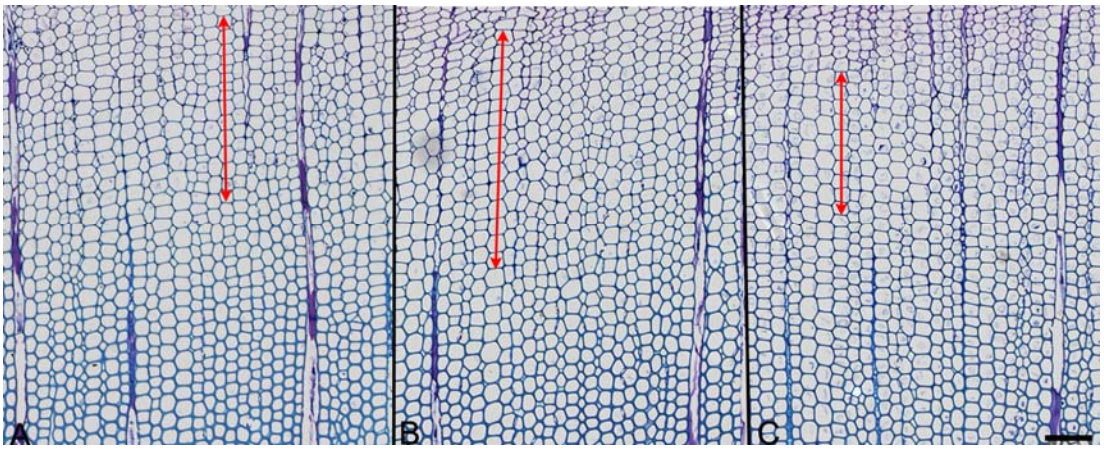


Figure. 6.5 *Transverse sections of cultured wood grown on low and high auxin concentrations stained with toluidine blue were observed. The cultured wood in low (A) and high (B) auxin concentrations had more number of cells undergoing secondary wall development than control (C). Scale bar = 100µm*

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Analysis of the cell count data suggests that exogenous application of auxin led to an increase in cell division in the organ cultures. The cultures not only underwent successful periclinal division but the radial file continuity was also maintained. Similar observations have been reported in earlier studies (Zakrzewski, 1983; Savidge, 1993; Putoczki, 2006). There was an increase in the number of cells in all the three regions defined in most of the cases. In the current study there were about 10 to 15 new tracheids formed. This observation was supported in a concurrent study of organ cultures of radiata pine where 10 to 15 new tracheids formed (Putoczki, 2006), while Savidge (1993) observed an increase of 20 new tracheids in organ cultures of *Larix laricina*.

The removal of the auxin source in plants by various treatments decreased the endogenous auxin concentration and this lead to inhibition of cambial activity, which was restored by exogenous application of auxin (Tuominene *et al*., 1997). Hence, it seems that auxin is essential for the maintenance of cell division activity of the cambium (Savidge, 1983). For division and growth of most types of *in vitro* cultured plant, cells an external source of auxin is required (Petrášek *et al.*, 2002). Supplying organ cultures with auxin in the form NAA resulted in not only a viable cambium but enhanced cambial activity even at low auxin concentrations. For the *in vitro* cultures to respond the ratio of external to internal concentration is important (Petrášek *et al*., 2002). Therefore, it seems that in this study even in low auxin concentration conditions the ratio difference was enough to cause an increase in the cambial activity. There is a general agreement in the literature that high auxin concentration increases number of tracheids significantly (Zajaczkowski, 1973; Zakrzewski, 1983; Kalev & Aloni, 1999). However, there were two instances where lower numbers of cells were observed at the high auxin concentration of 3 mM. Petrášek *et al.* (2002), in their culture increased the auxin concentration 10 fold the normal concentration and found that it led to prevention of cell division activity by the cambium. They suggested that when the internal auxin concentration rises above a critical threshold value then it inhibits the cell division. This could be one of the reasons for lower cell division in some of the higher auxin concentration organ cultures. Uggla *et al*. (1998), states that when exogenous auxin was supplied, the resulting internal auxin levels correlated well with the cambial growth response and this was observed overall in the current study.

6.3.7 Increased auxin concentrations tend to increase cell size of cultured wood

The light microscopy observations of the transverse sections of the cultured wood grown in different auxin concentrations gave the impression that the size of the cells had a tendency to be larger compared to the control. In order to confirm this, cells were measured using an image analysis programme (Image Pro Plus). The cells were measured with respect to their lumen area, radial and tangential length and cell wall thickness. The cells in the cambial region, developing cell region and the cells that already existed at the start of the culture (existing cells) were measured (method in section 6.2.19).

6.3.7.1 Cell lumen area

Measurements from the three cell regions were compared to the control. Across the three sets, only the cell lumen area of the cambial cells shows a clear trend when the results of the three sets were averaged. With application of exogenous auxin, the cambial cells tend to have a larger cell lumen area. However, this was not the case for developing cells region and the existing cells. In two sets (Set 1[a] and Set 2[b]), cells growing in the lower auxin concentration (0.003 mM) had the biggest cell lumen area in the cambial region.

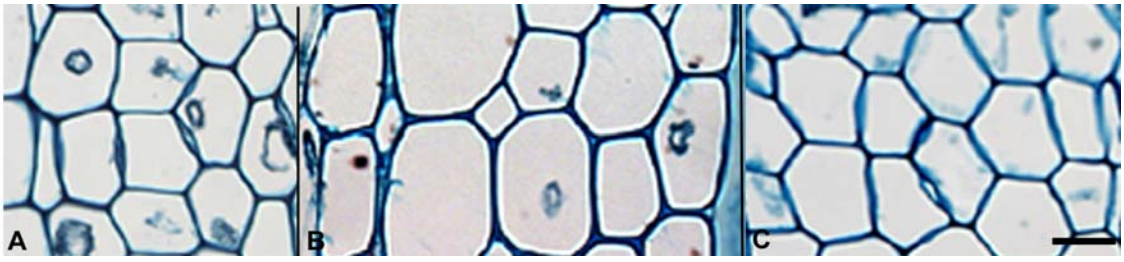

Figure. 6.5 *Transverse sections of cultured wood grown on low and high auxin concentrations stained with toluidine blue were observed. The developing cells region of the cultured wood in low (A) and high (B) auxin concentrations in some cases had cells with bigger cell lumen area than control (C). Scale bar = 100µm*

At the same time, there were two sets (Set 2^b and Set 3^c) with the smallest cell lumen area when grown in high auxin concentration (3 mM). The cells that already existed at the time of culture were not expected to change, as these cells seemed to have undergone secondary wall formation. However, there was change observed in these cells. It is likely that lignin is still being deposited in the cell walls, so there could be alterations in the lignification of these cell walls and this could affect the measurement of the cell lumen area during image analysis. The cell lumen area measurement, ranged between 518 to 1052 µm. Putoczki (2006) reported a similar observation and range of measurements in the radiata pine organ cultures, where the cell lumen area ranged between 497 to 1008 µm.

Auxin has been shown to stimulate cell expansion in many experimental systems and tissues that have been used for studies of plant growth (Tuominen *et al*., 1997). In an experimental study, *Pinus silvestris* was decapitated leading to a decrease in the endogenous auxin levels that caused an instant arrest in cambial activity as well as radial enlargement of the cells (Wodzicki & Wodzicki, 1973). In some studies, it has been pointed out that cambial derivatives would continue to expand as long as they were positioned within the field of significant auxin concentration. The studies also showed that the auxin gradient modulates the radial width of the xylem elements by regulating the duration of their expansion (Uggla *et al*., 1998; Sundberg *et al*., 2000). Therefore, it seems that under the influence of exogenous auxin the cultured wood developed cambial cells with larger cross- sectional area and an increase in radial dimension of xylem compared to the control.

6.3.7.2 Changes in radial and tangential length

Radial length

The cells were measured to study the changes in the cell dimension, to determine along which direction the cells changed the most when exposed to different auxin concentrations. The measurements from the image analysis data showed that there were more changes along the radial length of the cells than along the tangential. Along the tangential length in some instances, no change was observed. The cells in the cambial region were longest in the radial dimension in the cells exposed to lower auxin

concentration like 0.003 mM where an increase of 18% in the radial length was observed compared to the control, followed by 0.3 mM auxin concentration that showed 17% increase, and 3 mM auxin concentration showed a modest increase of 9% compared to the control.

Cultures grown in varying auxin concentrations						
	Set 1[a]		**Set 2[b]**		**Set 3[c]**	
Concentration levels mM	0.003,0.03	0.3, 3	0.003,0.03	0.3, 3	0.003,0.03	0.3, 3
Cambial region	High/Low 0.003mM biggest	High	High 0.003mM biggest	High/Low 3mM smallest	High/Low 0.03mM smallest	High
RE/Developing cells region	Low	Low	Low	Low 3mM smallest	High/Low	Low 3mM smallest
Existing region	Low	Low	low	Low	High	High 3mM largest
[a]Rotorua tree, initiated in april cultured for a period of one month. [b]Rotorua tree, initiated in April cultured for a period of two months. [c]Burnham tree, initiated in March cultured for a period of one month.						

Table. 6.3 *Summary of the cell lumen area observed in cultured wood grown on different auxin concentrations. When cell lumen area observed was higher than the control, it was highlighted. Increase in auxin concentration led to an increase in the cell lumen area observed in the cambial cells. (Based on measurements in Appendix 4. Statistical analysis in Appendix 4).*

The lowest auxin concentration of 0.03 mM showed the smallest increase of 0.7% in the radial length compared to the control. The radial dimension measurements ranged between 28 to 45 µm. These measurements were also in agreement with the previous studies where the range of radial length measurement was between 33 to 45 µm (Nyakuengama *et al*., 1999; Jackson & Nair, 2003) and the concurrent radiata pine culture study reported a range of 32 to 42 µm (Putoczki, 2006). No clear trend emerged in the developing cells region, and the cells that already existed in the organ cultures. In the existing cells, there was a tendency of the cultured wood to a decrease in the radial length compared to the control.

Tangential length

The tangential dimension measurements of all the cultures ranged between 23 to 30 µm. These measurements were similar to a previous study of radiata pine and a concurrent study done on the organ cultures of radiata pine wood (Skinner *et al*., 2003; Putoczki, 2006). These studies have reported a range of 23 to 30 µm and 24 to 32 µm respectively.

Cultures grown in varying auxin concentration						
	Set 1[a]		**Set 2**[b]		**Set 3**[c]	
Concentration levels mM	0.003,0.03	0.3, 3	0.003,0.03	0.3, 3	0.003,0.03	0.3, 3
Cambial region (radial length L)	High/Low	High	High	High	High/Low	Low
Cambial region (tangential width)	Low	Low	High/Low	Low	No change/ Low	High
Developing region (radial length)	Low	Low	High/Low	High/Low	High/low	High
Developing region (tangential width)	No change	No change	No change/ Low	Low	High/low	Low
Existing region (radial length)	Low	Low	Low	Low	High	High
Existing region (tangential width)	Low	No change	No change/ Low	High/Low	High	High

[a]Rotorua tree, initiated in april cultured for a period of one month.
[b]Rotorua tree, initiated in April cultured for a period of two months.
[c]Burnham tree, initiated in March cultured for a period of one month.

Table. 6.4 *Summary of the radial and tangential length observed in cultured wood grown on different auxin concentrations. When a radial length increase was seen compared to the control, it was highlighted. Application of exogenous auxin led to changes in the radial length (RL), while in the tangential width (TL) there were instances of no appreciable change between control and cultured wood. (Based on details of the measurements in Appendix 4, statistical analysis for same data in Appendix 4).*

Previous work has shown that exogenous application of auxin increased the xylem width and tracheid size (Little & Sundberg, 1991). This notion finds support in the observation made in fast growing trees the wood displayed a wider zone of expansion and tracheids

with wider radial diameter compared to the slow growing trees (Uggla *et al*., 1998; Sundberg *et al*, 2000). Auxin has been implicated in the stimulation of radial expansion and increase in radial diameter (Sundberg *et al.*, 2000). Hence, in the organ culture there was usually a tendency for the cells to increase in the radial diameter, particularly the cambial cells. However, in some cases a reduction in the radial diameter was also observed. According to Sundberg *et al*. (2000), a positive correlation between auxin concentration and radial diameter of tracheids was observed upto a certain threshold level, beyond which the auxin became inhibitory. Thus, it is quite possible that in some of the cultures the internal auxin concentrations became supra-optimal and led to a decrease in cell diameter. The radial width of the xylem cells can also be affected by the rate of cambial growth. It has been observed that with increase in cambial growth duration of cell expansion would be affected due to increased flow of cells through the cell expansion region (Sundberg *et al.*, 2000). In the cultured wood overall, there was an increase in the cambial activity (Table. 6.2 and 6.4) and it appears that the higher number of cells being produced affected the radial diameter of the cells. It another study with a transgenic hybrid aspen a large cross-sectional lumen area of the xylem and a wider zone of fibre expansion were observed. However, this large cross-sectional lumen area was not accompanied by an increase in diameter (Tuominen *et al*., 1997). It has also been suggested that increased cell size was compensated by reduced frequency of cell divisions (Campanoni & Nick, 2005), so it seems that this kind of interaction could be at play as well in the organ cultures where the high cambial cell division rate could lead to smaller cells and, conversely if the cells expansion increased, then there was likelihood of decreased cell division. There have also been suggestions that auxin controls cell division and cell elongation through different pathways (Campanoni & Nick, 2005), and that there is uniformity in the mechanisms controlling the rate of growth processes in each developmental regions (Sundberg *et al.*, 2000). The differences that were seen in the cultured wood regions could thus be caused by differential auxin concentrations. This could be another explanation for the observation of the cultured wood cell size and cambial activity.

6.3.7.3 Increased auxin concentrations might lead to thin cell walls in developing cells region and thicker cell wall in existing cells

In the transverse sections of the cultured wood, the developing cells region and the existing cells were measured to study the changes in cell wall thickness. The measurements were done with the help of Image Pro Plus (method in section 6.2.20). The image analysis data indicated that the developing cells had a tendency towards thinner cell walls compared to the control. In comparison, the existing cells had an increase in the cell wall thickness in two sets (Set 1^{a}, Set 2^{b}) except in set three (Set 3^{c}) a decrease in the cell wall thickness compared to the control was observed. The cell wall thickness of existing cells when grown in high auxin concentration of 3 mM increased by nearly 10% compared to the control.

Exogenous auxin promoted wall thickening (Wodzicki & Zajaczkowski, 1974). Hence, the thick walls that were observed in the existing cells were probably still undergoing secondary wall maturation when the organ culture was initiated, and the presence of exogenous auxin in the medium caused the cell walls to become thicker. Secondary wall development in the presence of exogenous auxin has been attributed to an increase in both rate and duration of wall thickening (Denne & Wilson, 1977; Porandowski *et al*., 1982). Supra-optimal concentrations of exogenous auxin induced the formation of thick walled, round tracheids (Little & Savidge, 1987). These findings suggest that the existing cells were affected by the exogenous auxin, which caused the thickness of the cell walls to increase.

At the same time, the developing cells showed thinner walls. It is likely that these cells were undergoing cell wall extension. Hence, the cell walls were thinner compared to the control. According to Wodzicki (1971), tracheid differentiation involves phase of enlargement followed by phase of maturation when the secondary wall is formed and that both these processes were independent of each other. It is therefore reasonable to assume, that both the regions were affected in a different manner by the exogenous application of auxin. Hence, the differences in cell wall thickness. The size of the cell and thickness of its secondary wall depend on the duration and rate of the phases of wall extension and secondary wall thickening. Tracheid wall thickness is apparently determined more by duration than by the rate of thickening (Wodzicki, 1971). It is quite possible then that the

developing cells in the presence of exogenous auxin have longer duration of cell wall extension and so the thinner cell walls.

Cultures grown in varying auxin concentrations						
	Set 1[a]		**Set 2[b]**		**Set 3[c]**	
Concentration levels mM	0.003,0.03	0.3, 3	0.003,0.03	0.3, 3	0.003,0.03	0.3, 3
Developing cells region	Low	Low	High	Low	Low	High/Low
Existing region	High	High	High	High	Low	Low
[a]Rotorua tree, initiated in april cultured for a period of one month. [b]Rotorua tree, initiated in April cultured for a period of two months. [c]Burnham tree, initiated in March cultured for a period of one month.						

Table. 6.5 *Summary of the cell wall thickness observed in cultured wood grown on different auxin concentrations. The cell wall thickness of the developing cells was thinner, where as the existing cell had thicker cell walls in the presence of exogenous auxin. In set 3 the cell walls were thin in both the regions. The data has been highlighted to show a possible trend in the measurements compared to the control (Based on the details of the measurements in Appendix 4. Statistical analysis in Appendix 4).*

6.3.8 Cultured wood showed to higher lignin levels in the cell wall layers

The results of the histochemical studies indicated that the lignin content increased in the cell walls of the tracheids in a dosage dependent manner. Hence, the wood growing in high concentration of auxin displayed more lignin levels in the cell wall as compared to the low auxin and the control wood.

6.3.8.1 Light and epifluorescence microscopy analysis

Investigations were carried out to see if there were any differences in the lignin content of the cell wall layers. The lignin in the cultured wood was visualised using safranin-fast green stain (section 3.2.3.2) and lignin autofluoresence at 280nm (section 4.2.4.2). The image analysis of the epifluorescence microscopy and the observations of the safranin-fast green stain indicated that the lignin content in the existing cell wall was higher than control (Figure. 6.7).

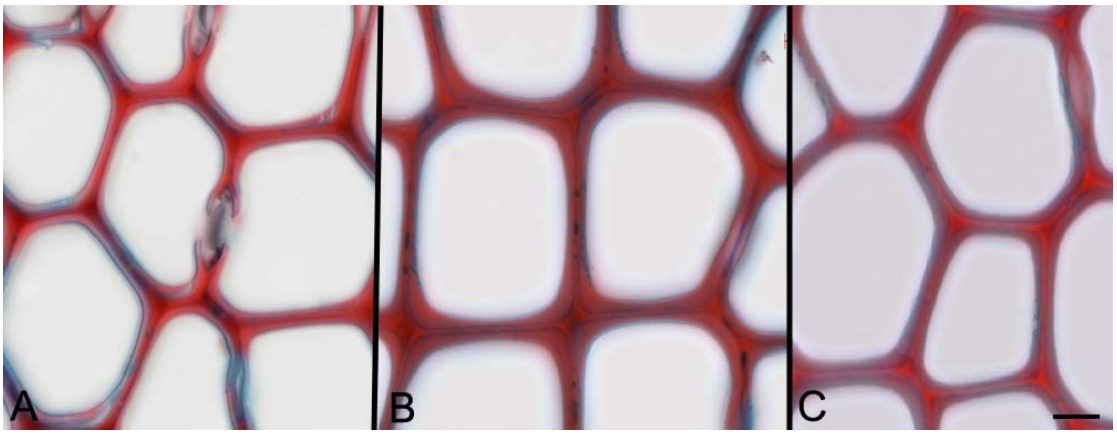

Figure. 6.7 *Transverse sections of cultured wood grown on low and high auxin concentrations stained with safranin-fast green were observed. The cultured wood in low auxin concentration (A) displayed more lignin than control but lower than the cultured wood grown on high auxin concentration. The cultured wood grown on high auxin concentration (B) had higher lignin levels than low auxin cultured wood and control (C). Scale bar=10µm*

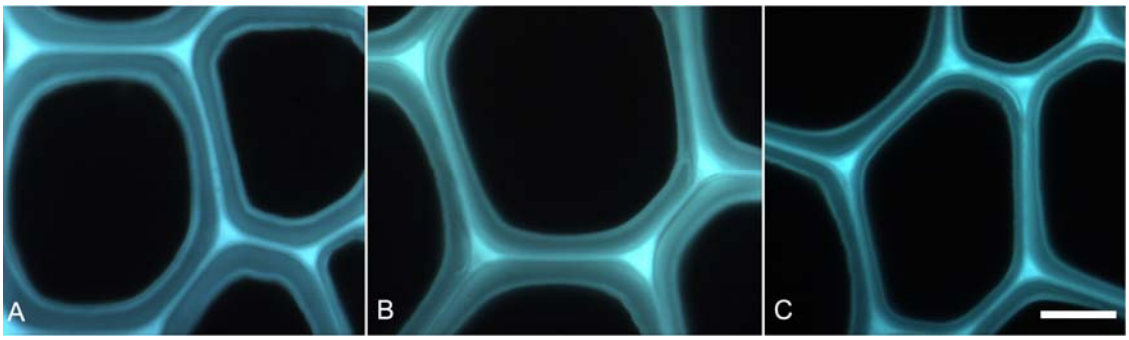

Figure. 6.8 *Transverse sections of cultured wood grown on low and high auxin concentrations stained were observed using epifluorescence microscopy. The cultured wood in low auxin concentration (A) displayed more lignin than control but lower than the cultured wood grown on high auxin concentration. The cultured wood grown on high auxin concentration (B) had higher lignin levels than low auxin cultured wood and control (C).Scale bar=1mm*

The cultured wood under high auxin conditions shows deeper red stain with safranin indicated higher lignin content, also the intensity of the lignin observed with the epifluorescence microscopy (Figure. 6.8) showed similar result where more lignin was observed in the cell walls of the cultures growing in high auxin concentration. The cultured wood grown in lower auxin concentration also appeared to have a higher lignin concentration compared to the control. Thus, it seems that in the presence of exogenous auxin the wood responded by increasing the levels of lignin in the cell walls. (epifluorescence microscopy and safranin stain observation summarised in Table. 6.6).

Cultures grown in varying auxin concentration						
	Set 1[a]		**Set 2**[b]		**Set 3**[c]	
Concentration levels mM	0.003,0.03	0.3, 3	0.003,0.03	0.3, 3	0.003,0.03	0.3, 3
Existing cells Epifluorescence	High	High	High	High	High/Low	High
Existing cells Safranin	High	High	High	High	Low	High
[a]Rotorua tree, initiated in april cultured for a period of one month. [b]Rotorua tree, initiated in April cultured for a period of two months. [c]Burnham tree, initiated in March cultured for a period of one month.						

Table. 6.6 *Summary of lignin observed in cultured wood cell walls of existing cells grown on different auxin concentrations. The existing cell had higher levels of lignin in the cell wall layers when exposed to exogenous auxin (Appendix 4 had data from lignin image analysis, and statistical analysis for the data). The results were highlighted when there was increase in lignin observed compared to the control to see a possible trend.*

The TEM pictures of the cultured wood stained with potassium permanganate that identified the distribution of lignin in the cell walls (stains lignin Singh & Daniel, 2000), showed that the cultured wood grown in the presence of high auxin concentrations had a more lignified cml and S_1 layer compared to the control and wood grown on low auxin concentrations (Figure. 6.9)

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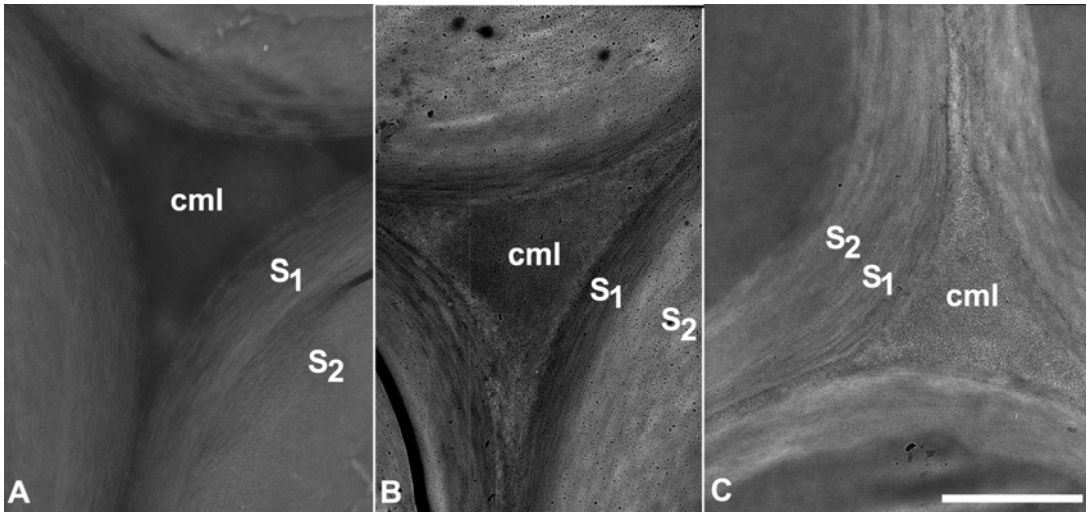

Figure. 6.9 *The cultured wood embedded in Spurr resin stained for detecting presence of lignin with the help of potassium permanganate were observed with TEM. The cultured wood had more lignin in the compound middle lamella and S_1 cell walls regions (A and B) compared to control (C). The cultured wood grown in high auxin concentration (B) had more lignin than the low auxin and control. Scale bar =2µm.*

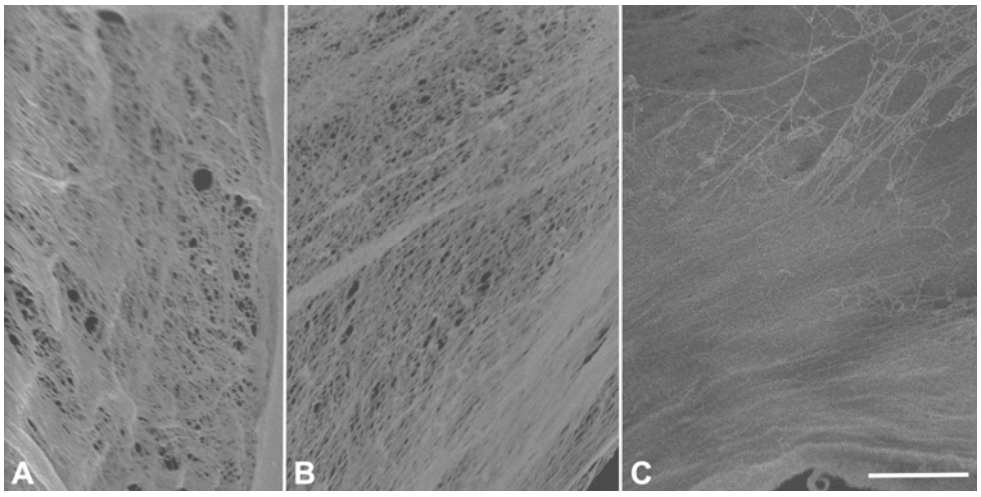

Figure. 6.10 *The cultured wood were observed with the help of FESEM. The cultured wood had more lignin content and when delignified with sodium chlorite there was a more loose network of cell wall layer observed and there seemed to be bigger pores in the cell wall of cultured wood (A and B)) compared to control. Scale bar =2µm.*

A trial was carried out to study the walls of the cultured wood using FESEM microscopy. Samples from only one set were observed in duplicate due to limitations of resources. The cell walls of control and low auxin concentration showed more compact and closer network of the cellulose microfibrils (Figure. 6.10) compared to cultured wood grown in higher auxin concentration. As the samples were delignified, the nature of the cell wall could be due to removal of lignin. Hence, the cell wall appears having a loose network, as there was no lignin in the cell wall to hold it together. Hence, it is possible that the lignin occupied more space in cultured wood grown in high auxin concentrations.

6.3.9 Biochemical analysis of auxin organ cultures

The microscopy observations indicated a higher level of lignification of the cell walls with increased in auxin concentrations. Biochemical analyses were also carried out to determine the lignin levels of cultured wood. Klason lignin assay, acetyl bromide assay and Py-GC-MS were carried out on cultured wood.

6.3.9.1 Klason and Acetyl Bromide lignin assay analyses

The Klason lignin analysis showed that the total lignin content in cultured wood was higher than the control.

Culture concentration levels	Acid insoluble lignin	Acid soluble lignin	Total lignin	Acetyl Bromide results
0.003 mM	25.85	1.04	26.89	20.62 ±4
3 mM	26.92	1.00	27.92	21.92 ±2.63
Control	24.2	0.95	24.2	20.4

Table. 6.7 *Klason and acetyl bromide lignin assay analyses are summarised in the table. The measurement was not repeated due to limitation of resources. The acetyl bromide assay was done on two sets of cultured wood and the values represent the average values. The values are percent lignin (w/w) and the data is ± standard deviation.*

However, this measurement was carried out only once on one set of control, hence, these results have to interpreted with caution. Acetyl bromide assay failed to show that there was more lignin content in the cultured wood compared to the control. The lignin values obtained with these analyses were in agreement with the concurrent study done on cultured wood (Putoczki, 2006).

6.3.9.2 Pyrolysis gas chromatography and mass spectrometry analysis

Lignin can be pyrolysed to produce a mixture of simpler phenols (Hatfield *et al*., 1991). Most of these phenols retain their substitution pattern from the lignin polymer and hence, can be used to identify the components from *p*-hydroxyphenyl, guaiacyl and syringyl moieties (Hatfield *et al*., 1991). For this analysis ground, cultured wood was sent to ensis, SCION (Rotorua, New Zealand) for lignin analysis by Py-GC-MS. This procedure was carried out on samples from one tree in duplicate, using wood from low and high auxin concentration cultures (section 6.2.21.3). The products of the pyrolysis breakdown that typically occur in radiata pine wood were identified (Appendix 4). Guaiacol, vinyl guaiacol, vanillin, eugenol, coniferyl alcohol and coniferyaldehyde were identified as pyrolysis breakdown products of lignin. The relative amount of the pyrolysis product was calculated by dividing the areas of all peaks in the chromatogram (Joly *et al*., 2000; Putoczki, 2006). Each of these pyrolysis products could provide a fingerprint that helps in characterizing the original sample (Joly *et al.*, 2000).

Lignin products	**Low auxin concentration**	**High auxin concentration**
Guaiacol	High	Low
Coniferyl alcohol	High	High
Vanillin	High	High
Coniferylaldehye	High	High
Phenol	High	High

Table. 6.8 *Summarised results of the changes observed in the lignin products analysed Py-GC-MS method. The cultures were explanted from same tree and grown for 2 months.*

The identified lignin products mostly seemed higher compared to the control. The cultured wood in the presence of auxin had higher lignin than control, except there was a decrease in the guaiacol product under the influence of high auxin concentrations. The coniferylaldehyde content was also higher than the control (one of the possible reason for the dark colouration in cultured wood.

Some carbohydrate products were also identified. 4-hydroxy-5,6-dihydro-(2H)-pyran-2-one is an indicator of xylan, it can also be derived from cellulose (Hatfield *et al.*, 1991), and anhydro gluco pyranose is a breakdown product of cellulose (Kelly *et al.*, 1991). The carbohydrate products analysed in the cultured wood showed differences compared to the control (Table 6.9). Carbohydrate products were all higher under the low auxin concentration conditions and for some high auxin concentration. However, there was lower content of 4-hydroxy-5,6-dihydro-(2H)-pyran-2-one in cultured wood grown on high auxin concentration compared to the control.

Carbohydrate products	**Low auxin concentration**	**High auxin concentration**
4-hydroxy-5,6-dihydro-(2H)-pyran-2-one	High	Low
HMF	High	No change
4-allyl phenol	High	No change
anhydro gluco pyranose	High	High

Table. 6.9 *Summarised results of the changes observed in the carbohydrate products analysed Py-GC-MS method. Carbohydrate content was higher under low auxin concentration compared to control and high auxin concentration. The cultures were explanted from same tree and grown for 2 months.*

The lignin analyses of the cultured wood with the microscopy indicate that exogenous auxin led to an increased lignin content of the cell wall layers of the wood. Some previous studies have reported similar results where the exogenous application led to an increase of active lignin formation (Aloni *et al*., 1990; Zhong & Savidge, 1995; Kano & Fukuoka, 1996). The cell walls thickness increased and the cell walls had higher lignin content when exposed to high auxin or auxin in combination with gibberellin

(Aloni *et al*, 1990). The results from the cultured wood studies find good agreement with these observations.

Another possible explanation for the increase in the lignin content could be stimulation of ethylene that could lead to an increase in lignin content of the cultured wood. Ethylene evolution stimulation by plant tissue in the presence of exogenous auxin is a well-established phenomenon (Romano *et al*., 1993). Ethylene production can also be induced in plants due to mechanical wounding (Sitbon *et al*., 1999). In the organ cultures, these conditions were prevalent; there was exposure of the tissue to exogenous auxin and there was mechanical wounding of the tissue during the explant preparation. Therefore, it seems highly likely that there would be evolution of ethylene in the organ cultures. There is considerable evidence that ethylene can activate enzymes that can lead to an increase in active lignin formation (Abeles *et al*., 1988; 1989). In transgenic plants over production of auxin stimulated simultaneous overproduction of ethylene (Romano *et al*., 1993). In the experimental study conducted by Sitbon and team (1999) high auxin levels increased the ethylene levels that in turn increased the lignin deposition. The same could possibly be happening in the cultured wood as well where the high auxin levels and the mechanical wounding could induce ethylene evolution leading to high lignin deposition in the cell walls of the cultured wood.

6.4 Summary

The results were interpreted with caution, as there was much variation both between and within the culture treatments (Appendix 4). Generally, experiments with exogenous auxin in both hardwoods and conifers have reported similar results to the current study, where the auxin was seen to affect most aspects of cambial growth in dose-dependent manner (Tuominene *et al*., 1997). Another concurrent study conducted on organ cultures of radiata pine also had similar results (Putoczki, 2006). According to Sundberg *et al*. (2000), auxin gradient functions as a morphogenetic field with the potential to give positional signalling in cambial growth, and that was the observation in the cultured wood, where the cells in the different developmental regions performed depending on the morphogenetic field of the auxin gradient. This was demonstrated by the high auxin concentration cultured wood displaying high radial width of developing

tracheids. The cultured wood also displayed altered lignification of the cell walls. The lignin content was higher in cultured wood compared to control. The exogenous auxin and mechanical wounding of the tissues could lead to increase in deposition of lignin in the cell walls. After studying the influence of auxin on xylogenesis of cultured wood, an interaction between auxin and boron (section 1.13) and their impact on xylogenesis was explored. The next chapter highlights some of the results obtained and examines the interrelationship between auxin and boron their influence on radiata pine xylogenesis.

Chapter 7

The interaction between auxin, boron and xylogenesis

7.1 Introduction

All vascular plants need boron (Fleischer *et al.*, 1998 and references therein) particularly plants that have well developed lignified xylem (Lewis, 1980). According to the previous studies, in higher plants boron is suggested to be involved in nucleic acid metabolism, responses to phytohormones, phenolic acid biosynthesis, cell wall metabolism, cell wall maturation, cell division and certain enzyme-mediated reactions (Dugger, 1973). Therefore, it seems that boron though a micronutrient, affects the development of plants in a big way. In New Zealand, the boron content of the soil is generally low and boron deficiency exists in a wide range of soils on eastern side of both islands (McLaren, 1993). Radiata pine plantations in New Zealand show some of the boron deficiency symptoms such as terminal bud death and permanent stem malformation, its absence has also been reported to be the cause of cracking, splitting, or checking (Skinner *et al*., 2003 and references therein). Hence, this study was set up with an aim to understand the role of boron in conjunction with auxin in radiata pine xylogenesis. Radiata pine was selected as a plantation species in New Zealand for its fast growth rate (McCurdy & Keey, 1999). It is well established that auxin is the primary phytohormone responsible for growth in plants, thus the assumption that radiata pine trees would have higher levels of auxin. High endogenous auxin levels might influence and even escalate the symptoms of boron deficiency (Coke & Whittington, 1968). This study was designed to not only understand the role of boron in xylogenesis, but also to explore the interaction between different concentrations of auxin and boron, and how they influence wood formation in radiata pine.

7.2 Material and Methods

7.2.1 Experimental Design

The modified organ culture method as described in chapter six originally used by Savidge (1993) and Leitch (1999) was used for this study to determine the interaction between auxin and boron and their effect on xylogenesis in radiata pine. The organ cultures were prepared from the radiata pine trees and grown on defined media with varying concentrations of auxin and boron. After a growth period of 30 to 60 days, the organ cultures were collected and subjected to various analyses to investigate the efficacy of the organ cultures and study the impact of auxin and boron on xylogenesis of radiata pine wood.

7.2.2 Tree material

Radiata pine trees growing on two sites were used for the study. Two trees from the Rotorua site owned by SCION, New Zealand, and four trees from the University of Canterbury Burnham site, New Zealand were used for this study. The trees were brought from the respective sites as described in section 6.2.2. The trees were stored at 4°C until the organ cultures were made from them within a week's time. The trees from the Burnham site were of the same clonal material (Clone 3). This was preferred to avoid any variations due to differences in the genotype. Clonal information was not available for the trees from the Rotorua site.

7.2.3 Culture media and its composition

The standard modified growth medium as described in section 6.2.3 (Appendix 3) was used for growing the organ cultures. The concentration of the phytohormone auxin and the micronutrient boron were varied in the current study in order to study the inter-relationship between the two and their impact on the xylogenesis of the radiata pine wood growing in the organ cultures.

The boron concentrations that were used for the study were 1 µM, 7 µM, 25 µM, and 100 µM. The boron concentration of 1 µm represents the lower boron concentration

and 7 µM the optimal level (that was grouped along with lower auxin concentration for the ease in the analysis). The boron concentration of 25 µM and 100 µM represents the higher boron concentrations where 25 µM could still be in the moderate range of boron concentrations whereas 100 µM represents the toxic levels. These boron levels were chosen based on the boron concentration in the standard media and those reported in the previous studies (Teasdale & Richards, 1990; Fleischer *et al*., 1998; Putoczki, 2006). The concentrations of boron less than 7 µM to 10 µM have been considered low (Teasdale & Richards, 1990; Fleischer *et al*., 1998). In the modified standard culture media, the boron concentration was at 16 µM (Putoczki, 2006). The auxin concentrations were also varied. The lower concentrations of auxin were 0.03 mM of NAA and higher concentrations were 0.3 mM and 3 mM of NAA. Four sets of growth media were prepared:

i) Auxin concentration was 0.03mM and the boron concentrations were 1 µM, 7 µM, 25 µM, and 100 µM.
ii) Auxin concentration was 0.3 mM and the boron concentrations were 1 µM, 7 µM, 25 µM, and 100 µM.
iii) Auxin concentration was 3mM and the boron concentrations were 1 µM, 7 µM, 25 µM, and 100 µM.

In this way, the influence of auxin and boron (low auxin+low boron, low auxin+high boron, high auxin+low boron and high auxin+ high boron) on wood formation could be studied in the organ cultures.

7.2.4 Preparation of the modified culture media

All the components of the standard modified culture media (section 6.2.3) were prepared in Nanopure water and for all the processes carried out during the preparation of the media either plastic or polypropylene containers were used so as to avoid any boron contamination from the glassware. The standard modified culture media (section 6.2.3) was divided into macronutrients, micronutrients, chelators and vitamins. All these groups of components were prepared separately in Nanopure water and stored at -20°C. The procedure followed for the medium preparation was the same as described by Putoczki (2006) in the boron organ culture study of radiata pine.

7.2.4.1 Controlling boron contamination

Precautions were taken to prevent contamination of the growth medium from water, chemicals and glassware by using polypropylene containers and boron free water (Dutta & McIlrath, 1964; Tanada, 1974; Putoczki, 2006). The mixture of components without any added boron was passed through an Amberlite IRA-743 ion binding column in order to remove any boron contamination (Bell *et al.*, 2002; Putoczki, 2006). In a trial study that was carried out to determine the efficiency of the column, culture medium (prepared without boric acid, sodium molybdate, potassium iodide or NAA that can bind to the column) was passed through two columns of Amberlite IRA-743 resin. The boron concentrations were measured before and after the passage through column (Putoczki, 2006). The medium collected after passage through the column had lower levels of boron (before 22 µM boron and after the column 9 µM boron in Putoczki, 2006). Hence, the Amberlite IRA-743 was adopted for removing the boron contamination from the chemicals (Figure. 7.1). For every litre of medium new columns were prepared (Putoczki, 2006).

Preparation of the ion-binding column

The ion-binding columns were prepared as described in (Putoczki, 2006). Plastic syringes (60 mL) were clamped on stands and were placed one above the other. They were connected to each other by plastic tubing with leur locks that controlled the flow of the mixture of medium components through the two syringes (see figure 7.1). At the bottom of the syringes, non-absorbent cotton was placed to prevent the resin from getting into the tubing. Resin slurry was made by combining the resin (Amberlite IRA-743) and Nanopure water; this was then poured into the syringes. Air bubbles were removed from the column by gently tapping along the sides of the column. The resin in the column was kept wet at all times and the medium was then passed through the column and collected in polypropylene containers.

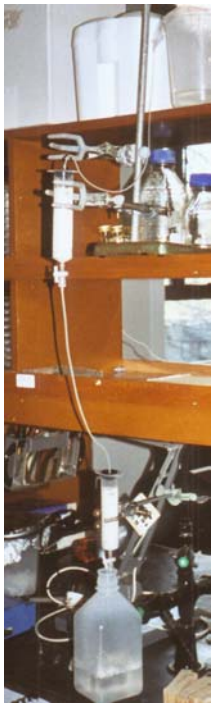

Figure. 7.1 *For the ion binding columns set up Plastic syringes filled with resin (Amberlite IRA-743) were clamped on stands and were placed one above the other and connected to each other by plastic tubing with leur locks that controlled the flow of the mixture of medium components through the two syringes. Picture courtesy T. Putoczki.*

Preparation of Agar

Agar (0.8% w/v. Difco Bacto-agar, 8,000 mg/L) was also prepared as described by Putoczki (2006), with ion binding resin to avoid any contamination of the growth medium. 8 g of agar and 25 g of ion binding resin (Amberlite IRA-743) were added to 250 mL boron free-Nanopure water. The solution was microwaved for approximately 30 minutes with periodic stirring. The resin beads were then removed from the molten agar by decanting and the agar was added to the medium prior to autoclaving.

This study was part of collaborative project between T. Putoczki and myself. The work varying boron concentrations in organ culture was carried out by T. Putozcki, while I carried out the interaction between auxin and boron on organ culture and the current chapter presents the results of the study conducted.

Preparation of boron free-Nanopure water

Nanopure water was passed through the ion-binding column and the boron free-water was then collected in polypropylene containers and stored at -20°C until its usage.

7.4.2.2 Preparation of the mixture of the components of the medium

The components of macronutrients, micronutrients, chelators and vitamins were added in appropriate volumes (Appendix 2) the preparation of the medium was done as per the protocol described by Putoczki (2006). myo-inositol (25 mg/L) and sucrose (2% w/v that is 20,000 mg/L) were weighed separately and combined with the rest of the components. The total volume was adjusted to 500 mL. The mixture of components was then passed through ion binding column to remove any boron contamination. Potassium iodide, sodium molybdate, manganese sulphate, magnesium sulphate, calcium chloride, boric acid and NAA can bind to the column (Matoh *et al*., 1992; Putoczki, 2006), hence, were added to the medium mixture after it had been passed through ion column separately prior to autoclaving the media. The stock solutions of potassium iodide, sodium molybdate, manganese sulphate, magnesium sulphate, calcium chloride, boric acid and NAA were prepared in boron free Nanopure water to control any contamination of the growth medium by these chemicals. Boron was also added to the growth medium in appropriate volumes to prepare the medium of desired concentration of 1,7, 25 and 100 µM. Agar as prepared earlier free of boron contamination was then added prior to autoclaving of the medium.

The pH of the medium was adjusted to 5.8 using HCl (1M; 0.1M) or KOH (0.1M). HCl and KOH were prepared in boron free-Nanopure water. The final volume of the medium was adjusted to 1 L and then autoclaved (121°C and 140kPa, for 20 minutes) in polypropylene containers. The sterilised medium was poured into pre-sterilised Petri-dishes (90mm diameter) to a depth of 3 to 4 mm, sealed with plastic film and stored in a dry place at room temperature.

7.2.5 Preparation of organ cultures

The explants were prepared from the radiata pine as described in section 6.2.4.

7.2.6 Culture growth conditions

In the current study, the organ cultures were grown at 21°C with 23.5 hours of light per day.

7.2.7 Growth period of the organ cultures

The organ cultures of radiata pine were allowed to grow for a period of 4 to 24 weeks and then collected and subjected to further analysis.

7.2.8 Fixation of organ cultures post culture growth

After duration of growth lasting for usually four to six weeks, the organ cultures were collected and fixed. The fixative was 4% formaldehyde in ¼ PBS (prepared as described in section 6.2.6.1).

7.2.9 Sample collection for analysis

Smaller pieces were cut from the organ cultures that have been stored in the fixative and used for further analysis. The sample collection was done only from the middle region of the organ cultures to avoid any edge effect in the samples collected.

7.2.10 Histochemical analysis

Similar to chapter 6 the organ cultures were subjected to histochemical analyses using safranin-fast green stain and toludine blue to study the wood anatomy and visualise the lignified tissues of the organ cultures. The wax sections were prepared from cultured wood for histochemical studies (section 3.2.2.1). The same protocols as described in section 3.2.2.2 was followed for the safranin- fast green staining of the slides and for toluidine blue staining the protocol as described in section 6.2.9.1 was followed. The stained slides were then observed using a Zeiss light microscope that was connected to a Zeiss Axioshop digital camera for collecting images.

7.2.11 Epifluorescence microscopy

The procedure as described in section 4.2.4.2 was used to observe lignin distribution in the cell wall layers of organ cultures. The dewaxed sections mounted in distilled water were observed using an Olympus lX 70 inverted microscope attached to a mercury lamp. Digital images were collected with a Cool Snap CCD camera (RS Photometrics) using standardised exposure conditions.

7.2.12 TEM microscopy of the cultured wood

The ultrastructure of the cell walls of the organ culture was studied using TEM. The protocols for sample preparation (section 6.2.12), fixation (section 6.2.13) and staining (section 6.2.14) were the same as those followed in chapter 6. The images were collected with a Hitachi H-600 transmission electron microscope at 75 kV or Phillips TEM at 80 kV (collected digital images). The negatives from Hitachi TEM were developed, scanned and collected (section 6.2.15).

7.2.13 Analysis of growth by cell count method

The same procedure as described in section 6.2.17 was used to determine if growth had occured in the organ culture.

7.2.14 Image analysis for lumen area, radial and tangential length

The image analysis procedure was the same as the one used previously and described in section 6.2.18.

7.2.15 Image analysis for cell wall thickness

The cell wall thickness measurements of the cells of the cultured wood were analysed using image analysis. The protocol described in section 6.2.19 was followed.

7.2.16 Analysis of the lignin content

The lignin content in the wood of the organ cultures was analysed using the Klason lignin method, acetyl bromide assay for lignin and Pyrolysis gas chromatography and mass spectrometry. The same procedure as described in section 6.2.20 was followed.

7.2.17 Analysis of lignin occupied area seen in cml/S_1

The area of lignin occupied in cell wall that was visualised by epifluorescence microscopy was also further analysed using Image Pro Plus (section 4.2.5, chapter 4). The measurements of the area occupied by lignin in cml/ S_1 were obtained and analysed.

7.3 Results and Discussion

7.3.1 Peripheral callus formation was observed in the cultured wood

The peripheral callus is a wounding response (Leitch & Savidge, 1995) hence, observed along the periphery of the organ cultures. The wounding would have occurred during the process of making explants. The callus was seen to appear along the periphery of the organ cultures within one week of culture initiation. These observations were similar to the ones made in the previous studies (Savidge, 1993; Leitch, 1999; Putoczki, 2006; section 6.3.3). When the boron nutrition was varied in the growth medium the callus that was formed initially was green in colour but towards the end of two months of period of growth the callus changed to brown (Putoczki, 2006). The cultures grown in the current study showed similar change of colour in callus.

Callus formation was taken as an indicator that the organ cultures were growing and responding to the treatments. However, similar to earlier studies no correlation could be established between the amounts of callus cambial cell division and tracheid production (Savidge, 1993; Letich, 1999; Putoczki, 2006; section 6.3.3).

7.3.2 Sub-culture and viability of the organ cultures

Sub-culturing of the organ cultures was attempted. However, like in the previous studies it was not successful (Savidge, 1993; Putoczki, 2006; section 6.3.4). The organ cultures once sub-cultured did not show any renewal in the formation of healthy callus. Similar to the previous study (section 6.3.4, chapter 6) the organ cultures were transferred after every four weeks onto fresh growth medium, once again no changes in the organ

cultures were observed with respect to callus formation, hence, it seems that the sub-culturing practice was not able to maintain the cultures for long period of time. Similarly, Savidge (1993) reported that the sub-culturing of the organ cultures was not possible and that cambial activity ceases in organ cultures after a period. There could be due to other growth factors necessary for cambium growth; once these are exhausted, the cambium could become dormant (Savidge, 1993).

7.3.3 Cultured wood had successful uptake of the micronutrients

In a concurrent study by Putoczki (2006), organ cultures were collected after two months of growth. The phloem layers and the xylem layers that were in contact with the medium were removed. The rest of the wood generally representing new wood (wood that had grown in culture) was ground to a homogenous powder and sent to for inductively coupled plasma-optical emission spectrometry/ mass spectrometry to R. Haslemore, Hill Laboratories (Hamilton, New Zealand) for analysis (Putoczki, 2006). This process was carried out to determine if there was successful uptake of the boron from the medium. The results showed that under high concentration boron conditions there was a significant increase in the boron present in the wood, however under low conditions it was difficult to infer the same (Putoczki, 2006). The boron levels showed a linear relationship between the boron concentrations in the medium to those found in the cultured wood after two months of growth (Putoczki, 2006; Figure. 7.3).

7.3.4 Cultured wood appeared darker than control

The cultured wood seemed to have wood characteristics quite similar to the earlywood of radiata pine and tracheid radial file continuity was maintained as well. Similarly, the other organ culture studies have shown the development of the earlywood under culture conditions (Zajaczkowski, 1973; Savidge, 1993; Putoczki, 2006; section 6.3.5) and the culture wood had radial file continuity of the tracheids (Savidge, 1993; Putoczki, 2006; section 6.3.5)

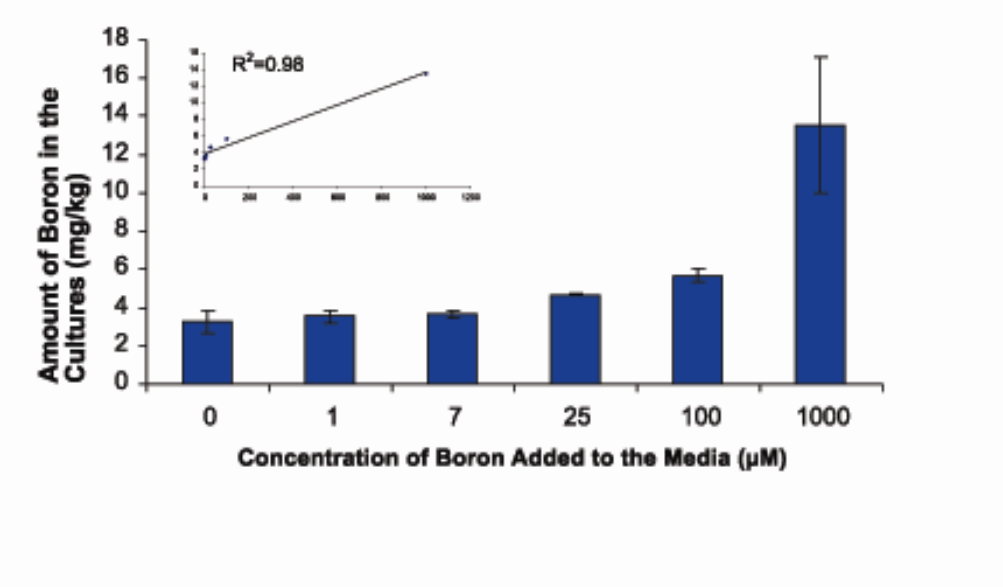

Figure. 7.3 *There was successful uptake of the micronutrient boron in the cultured wood (graph courtesy T. Putoczki).*

As in the previous studies cultured wood showed colour variation (Putoczki, 2006; section 6.3.5). The cultured wood had an orange-red to brown colour compared to the control. The colour of the cultured wood got darker as the auxin and boron concentrations increased in the medium (Figure. 7.3). The wood colour in the control explant was whitish in colour. The cultured wood grown under low auxin and low boron concentrations (0.03 mM and 1 µM and 7 µM) had a lighter coloured wood compared to high auxin and high boron (0.3 mM, 3 mM high auxin and 25 µM, 100 µM high boron). The cultured wood grown on higher auxin and boron concentrations showed that darkest colour wood. Overall, the colour of the wood seemed to have increased in a dosage dependent manner.

The colour changes were seen consistently in 14 sets of cultures observed. The observations were more likely to be due to culture response and not wounding response (Le Roux & Van Staden, 1991) as the change in colour was seen consistently in a concentration-dependent response. This observation finds support in similar findings reported earlier (section 6.3.5), and those observed in the concurrent study where the increasing in boron concentrations changed the colour of the wood (Putoczki, 2006). In the study conducted by Putoczki (2006), in organ cultures of radiata pine the boron

concentrations were varied (0 to 10,000 μM) with no change in the auxin concentrations (0.03 mM), it was observed that increase in boron concentration led to an increase in the colouration of wood from whitish in control to orange-red in cultured wood. One of the reasons for the changes in wood colour in the present study could be altered lignin content in the wood of the organ cultures. Hence, further study was carried out to determine the distribution of lignin in the cell walls and the content of lignin in the cultured wood.

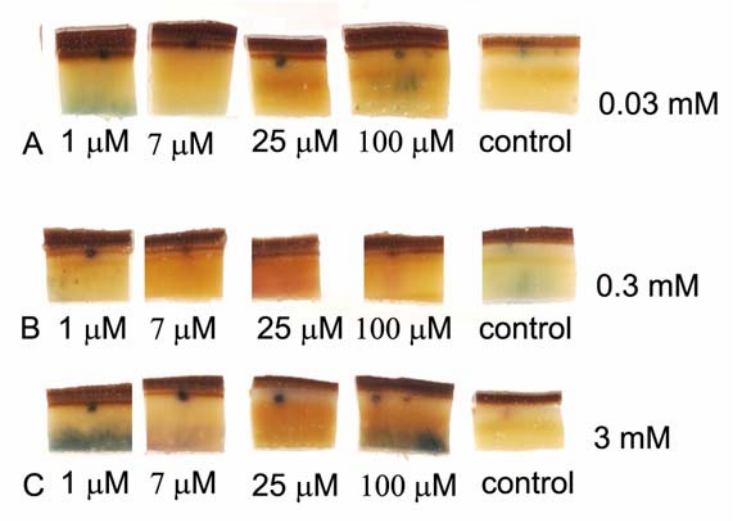

Figure. 7.3 *The cultured wood colour differed from control wood. There was an increase in colour of wood to darker shade of brown with increase in auxin and boron concentrations of growth media. The boron concentration is indicated in the bottom of the images and the auxin concentrations to the right of the images.*

7.3.5 The cell number increased with increase in auxin and boron concentrations

Transverse sections of cultured wood from six different trees and three sets from each treatment were observed for this study. The sections stained with toluidine blue were observed with the help light microscope and print outs of the images were collected. In each image, five radial cell files of cells were counted in the three defined regions of

cambial, RE and the developing region. The results summarised in Table. 7.1 (detailed measurements in Appendix 5).

The cambial activity showed an increase, in the cultured wood compared to control irrespective of the auxin or boron levels. However, when the number of cells in the cambial region of cultured wood grown on low boron concentrations (1 and 7 μM) growth medium was compared to those grown on high boron concentrations (25 and 100 μM), it was observed that the high boron cultured wood had more cells compared to the low boron concentrations (Appendix 5). The cells of RE region showed variability in the data. However, when the results were compared on an average with the control, between sets, then some pointers could be seen about the role of boron in xylogenesis. The interpretations were handled with caution, as there was variability in the data obtained. It was observed in two instances under the influence of low boron the number of cells undergoing radial expansion decreased on an average compared to the control, while their number increased when the boron concentration was increased. This was true for the two auxin concentration 0.03 mM and 0.3 mM (the increase was approximately 2% on an average compared to the control). The RE cells growing on high boron and high auxin concentration of 3 mM on the contrary showed a decrease in the number just like in the boron deficient condition. The cells of the developing region did not show any specific trend. However, in two instances once again there was a decrease in the number of cells under low boron concentrations (auxin levels were 0.3 mM and 3 mM) and an increase in the number of cells under high boron conditions and low auxin. In another parallel study conducted on the organ cultures of radiata, wood it was observed that under low boron conditions the number of cells decreased in the cambial and RE region, while, the number of cells increased in these regions in the presence of high levels of boron (Putoczki, 2006). Almost the same trend could be seen in the present study on exclusion of the data from the conditions of high auxin and low boron (discussed later in section 7.5), where the cell number decreased when boron concentration was low and increased when the boron concentration was increased irrespective of the auxin concentrations.

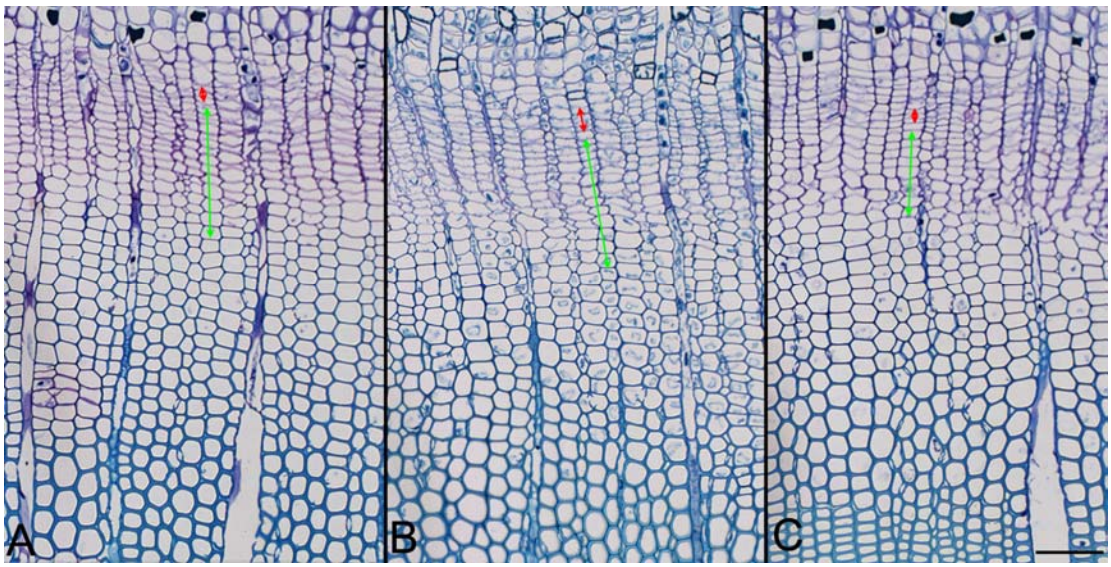

Figure. 7.4 *The cambial and the number of RE cells increased with increase in boron concentration (B) compared to the low boron concentration (A) and control (C).Scale bar=100μm.*

The results of the present study are supported by previous studies that have reported disruption of normal cell division under boron deficient conditions (Hu & Brown, 1994; studies cited by them). Some researchers have also pointed out that absence or lack of boron can lead not only to inhibition of growth but also to cessation of mitosis (Neales, 1964; Cohen & Albert, 1974). The impairment of the meristem tissue will finally lead to decreased cell division just as observed in the present study. Under the influence of low boron concentrations, there was usually a decrease in cell division and a lower number of cells produced though high auxin concentrations were high in some cases. Hence, it seems that for an increase in number of cells the levels of boron could be essential.

	Cambial		RE		Developing	
Boron levels (μM)	**1, 7**	**25, 100**	**1,7**	**25,100**	**1,7**	**25,100**
Set 1[a] (0.03mM)	High	High	High	High/Low	High	Low/High
Set 2 [b] (0.03mM)	High	High	Low	High	Low	High
Set 3[c] **(**0.03mM)	High	High	Low	Low	High	High
	Cambial		RE		Developing	
Boron levels	**1, 7**	**25, 100**	**1,7**	**25,100**	**1,7**	**25,100**
Set 1[d] (0.3mM)	High	High	High	High	Low	Low
Set 2[e] (0.3mM)	High	High	Low	High/Low	High	High
Set 3[c] (0.3mM)	High	High	Low	Low	Low	High
	Cambial		RE		Developing	
Boron levels	**1, 7**	**25, 100**	**1,7**	**25,100**	**1,7**	**25,100**
Set 1[f] (3mM)	High	High	Low/High	High	Low	Low/High
Set 2[b] (3mM)	High	High	Low	High/Low	High	High
Set 3[c] (3mM)	High	High	Low	Low	Low	Low/High

[a]Burnham tree, initiated culture in January, cultured for two months
[b]Rotorua tree, , initiated culture in April, cultured for one month
[c]Burnham tree, initiated culture in March, cultured for two months
[d]Burnham tree, initiated culture in March, cultured for two months
[e]Burnham tree, initiated culture in February, cultured for two months
[f]Rotorua tree, , initiated culture in March, cultured for two months

Table. *7.1 Summary of the number of cells observed in the cambial, RE and the developing region in the cultured wood compared to the control. The cambial activity increased in organ culture (detailed measurements in Appendix 5 and statistical analysis in Appendix 5).*

7.3.6 Cell size was altered in cultured wood compared to control

The light microscopy observations of the transverse sections of the cultured wood grown on different auxin concentrations gave an impression that the size of the cells was altered compared to the control. In order to confirm this, cells were measured using the image analysis programme (Image Pro Plus). The cells were measured with respect to their lumen area, radial length and tangential length and cell wall thickness. The cells in the cambial region, RE region and the cells that already existed at the time of the culture (existing cells) were measured and the data analysed.

7.3.6.1 Cell lumen area showed changes with change in auxin and boron concentrations

Transverse sections stained with toluidine blue were used for this study. Three sets of organ culture repeats were used for each treatment that was prepared from six different trees. The light microscopy images collected were analysed using Image Pro Plus software (method described in section 6.2.18). No distinct trend becomes visible from the analysis of the measurements of the cell lumen area for each treatment. Though there are variations in the data, there were some pointers as to the role of boron in xylogenesis. When the organ cultures were grown under the influence of low boron concentrations (1 and 7 μM) and lower auxin concentrations there was an increase in the cell lumen area in the cambial cells by 10% (comparing these treatment measurements with control). Similarly, the cell lumen seemed to have increased under the influence of low boron and high auxin concentrations (0.3 and 3 mM) by 4% compared to the control.

There were changes seen in the cultured wood grown on high boron and varying auxin concentrations as well. The cambial cell lumen area seemed to decrease in the presence of high boron concentrations (25 and 100 μM) and auxin concentrations of 0.03 mM and 0.3 mM.

When the cultured wood was grown on high boron and the highest auxin concentration (3 mM) there was an increase in the cambial cell lumen by 7% on an average compared to the control. However, the developing cells growing on high boron and highest auxin concentration of 3 mM had smaller cell lumen area. The existing cells cell lumen area was also altered. Generally, in all the high boron treatments irrespective of the auxin concentration there was decrease in the cell lumen area that was observed, though it was a small decrease.

The existing cells were not expected to change, however, measurements suggest otherwise. There is high possibility that these cells are still undergoing cell wall maturation and the changes during cultured growth could be affecting the cell wall thickening (mainly due to lignification; section 6.3.7.1).

	Cambial		Developing		Existing	
Boron levels	**1, 7**	**25, 100**	**1,7**	**25,100**	**1,7**	**25,100**
Set 1[a] **(**0.03mM auxin**)**	High	High	High	High/Low	High	High
Set 2 [b] **(**0.03mM auxin**)**	No change/Low	Low	High	High	Low	Low/High
Set 3[c] **(**0.03mM auxin**)**	High/Low	Low/High	Low	Low	High/Low	High/Low
	Cambial		New secondary wall		Existing	
Boron levels	**1, 7**	**25, 100**	**1,7**	**25,100**	**1,7**	**25,100**
Set 1[d] (0.3mM)	Low/High	Low	High	Low/High	Low	High/Low
Set 2[e] (0.3mM)	High/Low	High	High/Low	High	High	Low
Set 3[c] (0.3mM)	Low/High	High/Low	Low	Low	Low	Low
	Cambial		New secondary wall		New secondary wall	
Boron levels	**1, 7**	**25, 100**	**1,7**	**25,100**	**1,7**	**25,100**
Set 1[f] (3mM)	Low	Low/No change	Low	Low	High	Low/High
Set 2[b] (3mM)	High	High	High	Low	Low/High	Low/High
Set 3[c] (3mM)	High/Low	Low/High	Low/High	Low	High/Low	High/Low

[a]Burnham tree, initiated culture in January, cultured for two months
[b]Rotorua tree, , initiated culture in April, cultured for one month
[c]Burnham tree, initiated culture in March, cultured for two months
[d]Burnham tree, initiated culture in March, cultured for two months
[e]Burnham tree, initiated culture in February, cultured for two months
[f]Rotorua tree, , initiated culture in March, cultured for two months

Table. 7.2 *Summary of the cell lumen area observed in the cambial, the developing and the existing cell regions in the cultured wood compared to the control. The developing cells seemed to be most affected with change in boron concentrations (detailed measurement, and statistical analysis in Appendix 5).*

The alteration in cell wall thickness as a response to cultured treatments could be resulting in the image analysis measurements. Similar changes in the existing cells have been reported in the concurrent study conducted on organ cultures of radiata pine (Putoczki, 2006). The cell lumen area ranged between 324 to 1286 µM in the present study, and within the range of previous studies (section 6.3.7.1; Putockzki, 2006)

Boron deficiency is associated with disruption of expansion during periods of rapid organ growth (Hu & Brown 1994, Fleischer *et al*., 1998; Ghanati *et al*., 2001, Takano *et al*., 2001). Hence, in the present study the change in the boron levels were more appreciable in cells of the developing region as these cells could be still expanding and the levels of boron could affect their development. In the present study, the cells that were undergoing radial cell expansion under low boron concentrations displayed a decrease in the cell lumen area. Skinner and team (2003) have reported similar observations where they found the boron deficient radiata pine trees had small cell lumen compared to those fertilised with boron.

Even when the cultures were supplied with high auxin concentrations, there was a decrease in the cell lumen when the levels of boron were low. In the previous study (section 6.3.7.1), the cultured wood mostly had bigger cell lumen area when grown under the influence of high auxin. However, in the current study even with the high auxin present in the media the cell lumen area was smaller for the cells under boron deficient conditions. The process of cell enlargement as per earlier reports requires higher levels of boron than that required for cell division (Fleischer *et al*., 1998). Therefore, it seems that the boron levels in the cultured medium were not sufficient to facilitate normal cell expansion. Even the high auxin concentrations could not compensate for the boron deficiency; thus, a decrease in cell lumen area was observed in most of the boron deficient cultured wood.

7.3.6.2 Radial and tangential length

The transverse sections stained with toluidine blue were used for this study as well. The images were analysed using Image Pro Plus. The analysis of the data showed that there was variability both within and between sets of the cultured wood studied. However, on an average there were some trends that could help in making deductions about the role of boron and auxin in xylogenesis of radiata pine wood.

Radial length

On an average when the boron concentration was low (1 and 7 μM) and the auxin concentration was 0.03 and 0.3 mM there was an increase in the radial length of the

cambial cells by 15% in both the cases. However, there was a decrease in the radial length with increase in auxin concentration (3 mM) with low boron concentration by 3% compared to the control. In the cells of the developing cells and the existing cells region, the radial length decreased under the influence of low boron concentrations. Hence, it seems that the cells generally have a tendency to have smaller radial length when grown in boron deficient conditions.

Under the influence of high boron, conditions (25 and 100 μM) irrespective of the auxin concentrations the cambial cells increased in the radial length compared to the control. The cells in the developing region also increased in radial length compared to control in the presence of high boron conditions except when the auxin concentration was 3 mM there was a decrease of 8% on an average compared to control. The existing cells with high boron and high auxin concentrations (0.3 and 3 mM) showed a decrease in the radial length of the cell.

Tangential length

When the data was analysed for the tangential length of the cells measured in the three defined regions once again there was variability in the data and no distinct trends emerge. On an average, though there were some differences that become apparent. The changes that occur in the tangential length were in the range of 2 to 10% compared to the control. The cambial cells of the cultured wood grown on low boron (1 and 7 μM) and auxin concentrations of 0.03 and 0.3 mM on an average showed lower tangential length. Similarly, the cambial cells of cultured wood growing on high boron concentrations (25 and 100 μM) on an average also showed decrease in tangential length irrespective of the auxin concentration. However, the cambial cells of cultured wood grown on low boron and 3 mM auxin concentration showed an increase in the tangential length on an average of 6% increase compared to the control.

The tangential length of the cells in the developing region tends to decrease under low boron concentrations irrespective of the auxin levels in the growth medium. The tangential length under high boron concentrations (25 and 100 μM) and low auxin (0.03 mM) showed an increase compared to the control. There were no clear trends that become apparent in the case of existing cells. However, in most of the cases, the

tangential length in the cells remained without much change and if there was a change then there was usually a decrease in tangential length when boron levels were high and the tangential length showed an increase in the low boron conditions irrespective of auxin levels on an average when compared to the control.

In the literature there were both type of cellular activity cited for cells growing under boron deficient condition. According to Fleischer *et al*., (1998) cells are larger under boron deficiency, whereas Ishii *et al.,* report smaller cells (2001). Hu and Brown (1994) stated that the expanding tissues in boron deficient conditions have uneven and disorganised cell expansion. In the present study, similar observations were made, there were changes observed, in either radial length or tangential length or along both the lengths in some cells observed. Boron cells were reported to be shorter by 70% in longitudinal and 20% in the transverse directions (Ishii *et al.*, 2001). Usually with decrease in boron, the cells of the cultured wood also showed a decrease in size irrespective of auxin concentration. However, the cambial cells were seen to increase in radial length even in the low boron concentrations. This behaviour of the cambial cells under boron has been reported earlier by Palser and McIrath (1956) who observed that the cambial cells stretched under boron deficiency. In the current study, too cambial cells had increased along the radial length mostly under the low boron conditions.

7.3.6.3 Cell wall thickness

The light microscopy images were analysed further using image analysis to measure the cell wall thickness of the cultured wood (measured as described in section 6.2.19). The cell wall thickness of the cultured wood grown in low boron conditions showed an increase in the cell wall thickness overall on an average compared to wood grown in on high boron conditions. These results were interpreted with much caution, as there was much variation both within and between culture treatments (Table. 7.4 and Appendix 5).

	Cambial		Developing		Existing	
Boron levels	**1, 7**	**25, 100**	**1,7**	**25,100**	**1,7**	**25,100**
Set1[a](0.03mM) Radial	High	High/Low	High	High	High/Low	High
Set1[a](0.03mM) Tangential	High	No change/High	High	High	High	No change
Set2[b](0.03mM) Radial	High	High/Low	No change/High	High	Low	Low/No change
Set2[b](0.03mM) tangential	Low	Low	High	High	High	No change
Set3[c](0.03mM) Radial	Low/High	Low/High	Low	Low/High	No change/Low	High
Set3[c](0.03mM) tangential	Low/No change	Low/High	Low	Low/No change	High/Low	No change
Boron levels	**1, 7**	**25, 100**	**1,7**	**25,100**	**1,7**	**25,100**
Set1[d](0.3mM) radial	High	No change/High	High	High	Low	High/Low
Set1[d](0.3mM) tangential	Low	Low	Low	Low/High	Low	High/Low
Set2[e](0.3mM) radial	High	High	Low	Low	High	Low
Set2[e](0.3mM) tangential	High	High	No change	Low/High	High/No change	Low/No change
Set3[c](0.3mM) radial	Low/High	High/Low	Low	Low	Low	Low
Set3[c](0.3mM) tangential	High/Low	High	Low	Low	Low	Low/High
Boron levels	**1, 7**	**25, 100**	**1,7**	**25,100**	**1,7**	**25,100**
Set 1[f] (3mM) radial	Low	No change/High	Low	Low	High	Low/No change
Set 1[f] (3mM) tangential	Low	High	Low	Low	High	No change/High
Set 2[b](3mM) radial	High/Low	Low/High	Low/High	Low	Low/No change	Low
Set 2[b](3mM) tangential	No change	Low/High	High	No change	Low/High	Low
Set 3[c](3mM) radial	No change/Low	Low/High	Low/No change	Low	No change/Low	No change/Low
Set 3[c] (3mM) tangential	High	Low/No change	No change	Low	No change	High

[a]Burnham tree, initiated culture in January, cultured for two months
[b]Rotorua tree, , initiated culture in April, cultured for one month
[c]Burnham tree, initiated culture in March, cultured for two months
[d]Burnham tree, initiated culture in March, cultured for two months
[e]Burnham tree, initiated culture in February, cultured for two months
[f]Rotorua tree, , initiated culture in March, cultured for two months

Table. 7.3 *Summary of radial and tangential length observed in the cambial,*

the developing and the existing cell regions in the cultured wood compared to the control. Overall, it seems that low boron decreases radial length with increase in boron concentrations there was an increase in the cell lumen area irrespective of the auxin concentrations (detailed measurements and statistical analysis in Appendix 5).

When the data was analysed with regards to average values within the treatments then there were some differences that became apparent and were used to interpret the role of boron and auxin on cell wall thickness. Under low boron concentrations and with auxin concentrations at 0.03 mM and 0.3 mM the cell wall thickness increased compared to control in the developing region. The cells that already existed in the cell walls also showed changes in the cell wall as compared to the control.

	New secondary wall		Existing	
Boron levels	**1, 7**	**25, 100**	**1,7**	**25,100**
Set 1[a] (0.03mM)	High/Low	High/Low	High	Low
Set 2 [b](0.03mM)	High	High	High	High
Set 3[c] (0.03mM)	High	High	Low	Low
	New secondary wall		Existing	
Boron levels	**1, 7**	**25, 100**	**1,7**	**25,100**
Set 1[d] (0.3mM)	Low	Low/High	High	High
Set 2[e] (0.3mM)	High	High	High/Low	Low/High
Set 3[c] (0.3mM)	High	Low/High	Low	Low/High
	New secondary wall		New secondary wall	
Boron levels	**1, 7**	**25, 100**	**1,7**	**25,100**
Set 1[f] (3mM)	Low	Low	Low	Low
Set 2[b] (3mM)	Low	High/Low	High	High
Set 3[c] (3mM)	High	High	High	Low/High

[a]Burnham tree, initiated culture in January, cultured for two months
[b]Rotorua tree, , initiated culture in April, cultured for one month
[c]Burnham tree, initiated culture in March, cultured for two months
[d]Burnham tree, initiated culture in March, cultured for two months
[e]Burnham tree, initiated culture in February, cultured for two months
[f]Rotorua tree, , initiated culture in March, cultured for two months

Table. 7.4 *Summary of cell wall thickness observed in the developing and the existing cell regions in the cultured wood compared to the control. Overall, the existing cells showed increase in the cell wall thickness irrespective of the boron concentration (detailed measurement and statistical analysis in Appendix 5).*

Overall the thickness of cell walls of the existing cells observed had increased in all the treatments except in one where the boron levels were high and the auxin concentration was low (0.03 mM).

So it seems that the boron levels did not influence the existing cells, however the auxin levels influenced the existing cells and increased the cell wall thickness (as per the correlation made from the results of the previous study and the results of the current study). In the previous study (section 6.3.7.3 and Table. 6.5), it was observed that the existing cells of the cultured wood displayed an increase in the cell wall thickness in the presence of exogenous auxin. Similarly, in the current study in the low or high boron condition the cell walls show increase in cell wall thickness and the cells seem to be responding more to the exogenous auxin than to the varying levels of boron concentrations.

The changes that were seen in the developing cells seem to be more influenced by the varying levels of the boron concentrations. In the previous study, developing region cells showed usually thinner cell walls as compared to the control (section 6.3.7.3) whereas, in the present study there is an increase in the cell wall thickness observed in low boron conditions. This could be the response of the cultured wood cells to the boron deficiency in the growth medium. The observations made in the present study find support in some previous studies that observed boron deficient cell walls appear to be thicker (Lee & Aronoff, 1966; Hu & Brown, 1994). The thickness of the cell wall has been explained as the swelling of the cell wall, it was pointed out that the swelling of the cell wall was not due to increase in the density of the cell wall but due to the lack of cross-linking of borate and rhamnogalacturonan II (RGII) (Ishii *et al.*, 2001). RGII exists in all higher primary walls and is covalently cross-linked by borate and the boron functions as the cross-link for pectic polysaccharide RGII (O'Neill *et al.*, 2001). Hence, in boron, deficient condition there is lack of boron for the cross-linking and the cell wall appears swollen. Ishii et al., (2001), have reported such cells in their study where in the boron deficient conditions the cell walls were swollen compared to the thin walled normal cells. The disruption in the cell wall cross-linking can affect the normal

distribution of lignin in tracheary elements and this can lead to swelling of cellulose into matrix (Ishii *et al.*, 2001).

Therefore, it seems that the boron levels could be affecting the cells while they undergo development like the cells of the developing region, while the existing cells were more influenced by the auxin levels. According to O'Neill *et al*., (2001), boron functions in the plant primary walls predominantly. As seen in the current study the developing cells were influenced more by the boron levels, compared to the existing cells that have undergone their primary wall development and undergoing secondary wall development and lignifications that were influenced by the auxin levels and not by the boron levels. The following sections will highlight the work that was carried out to look at the ultrastructure of the cell walls and the lignification in the cell walls of the cultured wood.

7.3.7 Cultured wood tends to have higher lignin levels

The results of the histochemical studies indicated that the lignin content increased in the cell walls of the cultured wood compared to the control. Some of the sections that follow will highlight the results of these studies examining lignin distribution and content in the cultured wood.

7.3.7 1 Light and epifluorescence microscopy analysis

The transverse sections of cultured wood grown on varying concentrations of boron and auxin were subjected to light microscopy and epifluorescence microscopy to study the distribution of lignin in the cell wall of the cultured wood and the effect of boron and auxin concentrations on the lignification of the cell wall layers.

Light microscopy was carried out on safranin-fast green stained slides. The safranin stains the lignin red and fast green stains the cellulose blue in the cell walls of the wood (Johansen, 1940). The cultured wood treatments when compared to the control showed that the high boron treatments had more lignin compared to either control or the low boron condition (Figure. 7.4).

The cultured wood transverse sections were also observed using epifluorescence microscopy, as it is more recommended method for visualising lignin distribution in wood cell (Scott *et al*., 1969). The epifluorescence microscopy showed that there was

more areas of intense lignin autofluorescence seen in the in the low boron condition compared to the high boron condition (Figure. 7.5) although the cml/S_1 area in high boron condition gives an impression of being brighter. The cultured wood stained with safranin mostly showed the presence of low lignin levels in the cell wall of cultured wood grown on low boron concentrations while the cultured wood grown on high boron concentrations displayed high levels of lignin irrespective of the auxin concentrations (Figure. 7.4). Image analysis was carried out on the same epifluorescence microscopy images by picking the area of highest pixel intensity and measuring the total area of similar intensity observed in the cell walls of the cultured wood. The data obtained from the image analysis showed that cultured wood grown on low boron concentrations had more area of the cell wall occupied with lignin as compared to the high boron and control. These observations were contrary to the ones observed with the safranin-fast green stain. There were higher lignin levels in the cultured wood grown on low boron concentrations than on high boron concentrations.

A reduction in lignification with decrease in boron concentration was indicated by the safranin stain observation. A positive reaction with this reagent however does not necessarily indicate the presence of lignin in tissues, for several other commonly occurring cell constituents could also produce a red colour with this reagent. The safranin stain can give positive reaction with phenolic hydroxyl groups in general (Sarkanen & Ludwig, 1971). In the present study, however, this test was supplemented epifluorescence microscopy that is better indicator of lignin. The microscopy studies were also further supplemented by quantitative determinations to observe the lignin levels in the cultured wood.

7.3.7.2 TEM analysis

The cultured wood was also observed using TEM to study the ultrastructure of the cell wall and the lignin distribution in the cell wall layers. The cultured wood ultra thin sections stained with potassium permanganate (stains lignin in the cell walls, Singh & Daniel, 2000) were observed. The cml showed the darkest staining indicating presence of lignin, while the lignin in the S_1 layer of the secondary wall showed a striated appearance, as previously observed in the radiata pine wood (Singh *et al*., 2002).

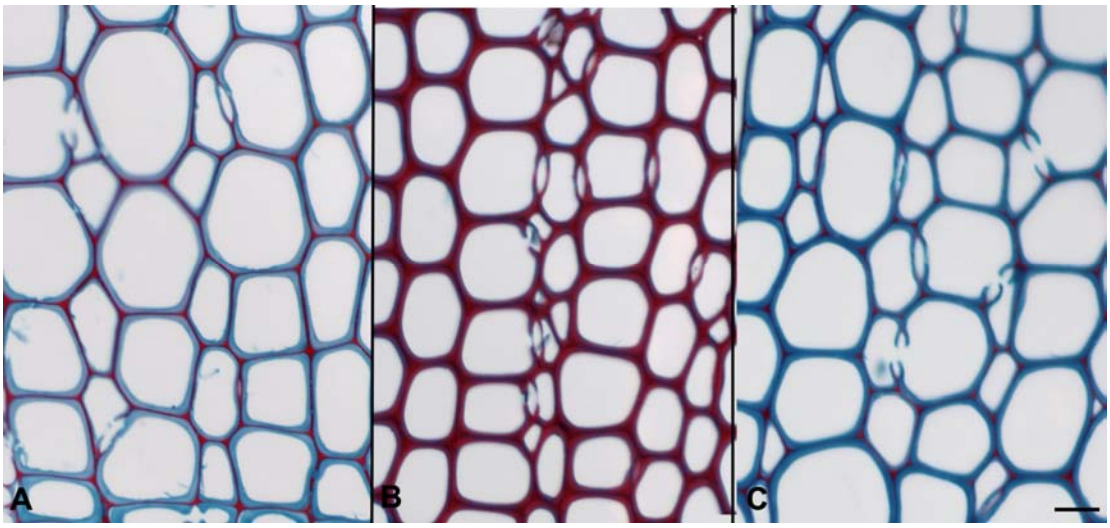

Figure. 7.4 *Transverse sections of cultured wood stained with safranin-fast green were observed. The cultured wood grown on low boron displayed lower levels of lignin (A) and when in the presence of high boron showed higher lignin levels (B) in the cell wall irrespective of the auxin concentrations compared to the control (C). Scale bar=20 µm.*

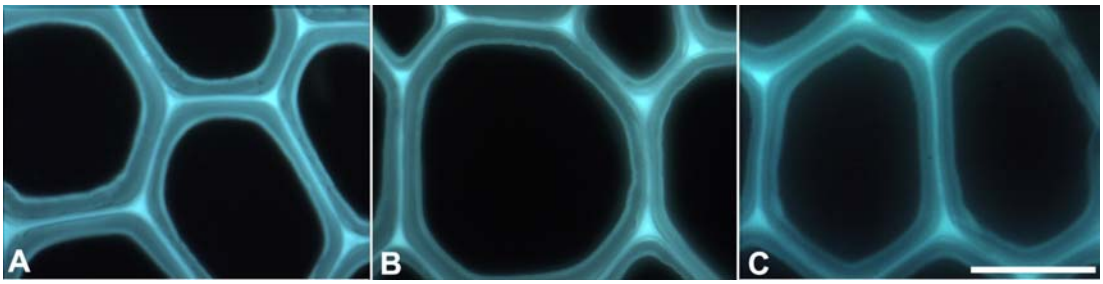

Figure. 7.5 *Transverse sections of cultured wood stained were observed using epifluroescence microscopy. The cultured wood grown on low boron displayed overall higher lignin levels in low boron concentrations (A) as compared to higher boron concentrations(B) irrespective of the auxin concentrations when compared to the control (C). Epifluorescence microscopy done at 100x magnification.*

It was seen that the cultured wood grown on low boron concentrations showed a somewhat larger area of the cml or a 'swollen' cml compared to the high boron concentration and control (Figure. 7.6). Ishii *et al*. (2001), have reported similar observations earlier where the boron deficiency led to swollen cell walls.

7.3.8 Biochemical analysis of the cultured wood

There were indications in the microscopy study that there were differences in the lignin levels in the cultured wood. Biochemical analysis was carried out on the cultured wood to confirm the microscopy observations. There were three lignin assays that were carried out, the Klason lignin assay, acetyl bromide assay and Pyrolysis gas chromatography and mass spectrometry.

7.3.8.1 Klason and acetyl bromide assay

Klason and acetyl bromide assay was performed on finely ground cultured wood. The samples for Klason lignin assay were sent to Veritec, SCION (Rotorua, New Zealand). The acetyl bromide assay was carried out by the protocol as described in section 6.2.20.4.

The lignin content measured in the present study was similar to the previous studies (section 6.3.9.1; Putoczki, 2006). Mostly the cultured wood seems to have higher lignin content compared to the control, except in 1 µM boron and 3 mM auxin concentrations the total lignin content was lower than the control. The data presented variability and no clear trend emerged. (Table. 7.4)

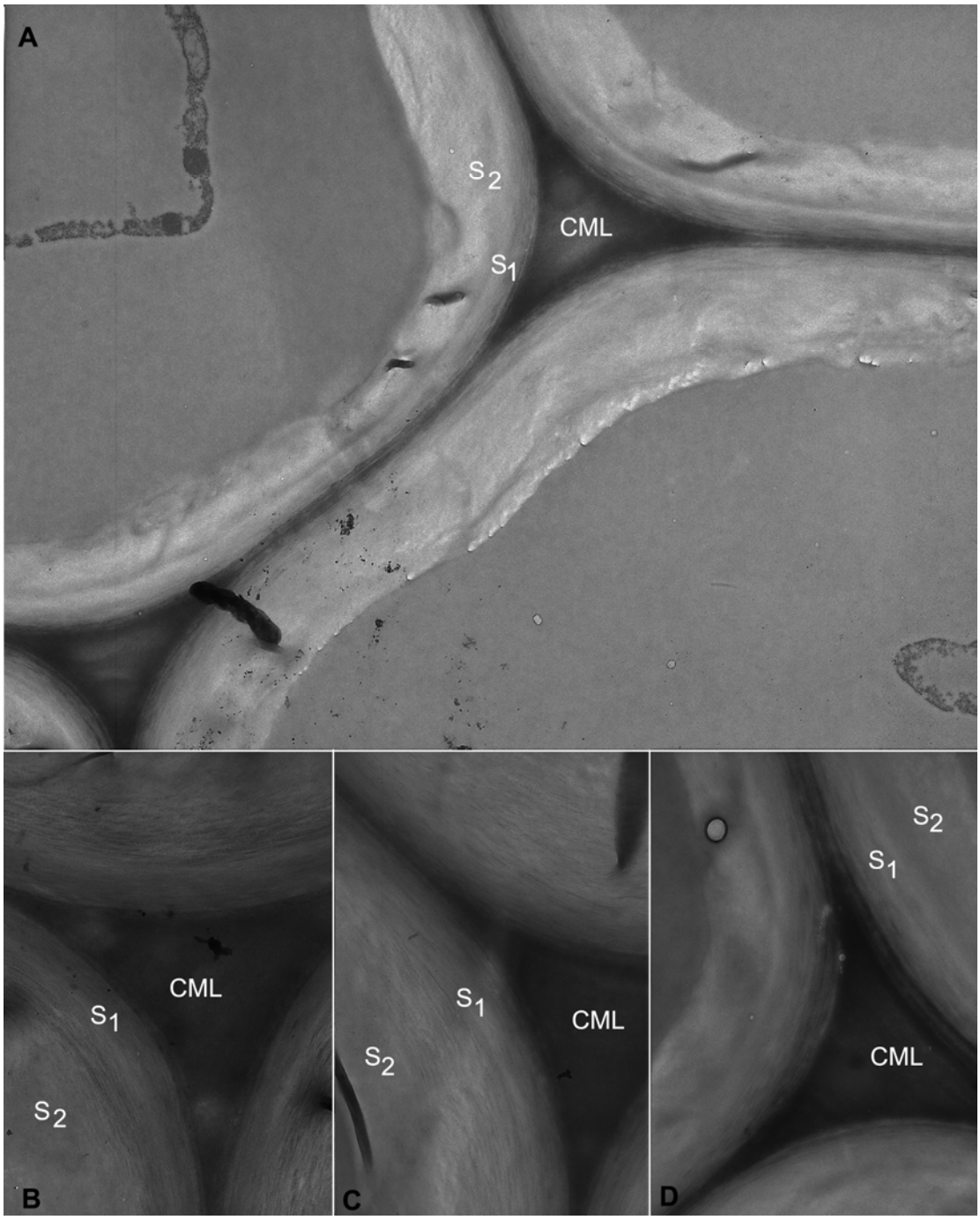

Figure. 7.6 *Transverse ultra thin sections of cultured wood stained with potassium permanganate were observed using TEM. The cultured wood grown on low boron concentrations (B) displayed overall swollen cml region as compared to higher boron concentrations(C) (irrespective of the auxin concentrations) and control (A and D).*

Culture concentration levels	Acid insoluble lignin %w/w	Acid soluble lignin %w/w	Total lignin %w/w	Acetyl Bromide results
1µM, 0.03mM	27.31	1.20	28.51	22.31 ±1.88
100µM, 0.03mM	24.49	1.15	25.64	25.71 ±0.14
1µM, 0.3mM	27.48	1.02	28.50	21.35 ±2.62
100µM, 0.3mM	27.70	1.01	28.71	20.41 ±3.81
1µM,3mM	19.80	1.16	20.96	27.75 ±9.59
100µM,3mM	25.38	0.97	26.36	20.95 ±1.61
Control	24.2	0.95	24.2	20.4

Table. 7.5 *Klason and acetyl bromide lignin assay was conducted on cultured wood. No clear trends emerge though the cultured wood seemed to have higher lignin content than control. The acetyl bromide assay was done in duplicate whereas the Klason assay due to limitations of resources was represents values of one time measurements.*

7.3.8.2 Pyrolysis gas chromatography and mass spectrometry

Finely ground cultured wood samples were sent to ensis, SCION (Rotorua, New Zealand) for Py-GC-MS. The measurements were carried out on the wood samples in duplicate on the low and high boron concentrations and the corresponding low and high auxin concentrations. Several products that are found typically in radiata pine were identified in the cultured wood during this analysis (detailed measurements in Appendix 5). There did not seem to be many variations in lignin content of the cultured wood with respect to the boron levels in the growth medium. However, they seem to be influenced by the auxin concentrations in the growth medium (compare Table.6.8 and Table. 7.6).

Lignin products	**0.03 mM auxin**		**0.3 mM auxin**		**3 mM auxin**	
Boron levels	**1 µM**	**100 µM**	**1 µM**	**100 µM**	**1 µM**	**100 µM**
Guaiacol	Low	Low	Low	Low	Low	Low
Coniferyl alcohol	High	High	Low	Low	Low	Low
Vanillin	High	High	Low	Low	Low	Low
Coniferylaldehye	Low	Low	High	High	High	Low
Phenol	High	High	No change	No change	No change	Low

Table. 7.6 *Summary of the changes observed in the lignin products identified by the Py-GC-MS analysis of the cultured wood. The lignin content in the cell walls of the cultured wood seems to be more influenced by the auxin concentration than the boron levels.*

The data presented may be able to indicate changes in the structure of lignin. However, it does not provide conclusive evidence for changes in the original lignin composition (quoting M. Jarvis pers comm. in Putoczki, 2006). Earlier studies indicate that boron deficiency can lead to accumulation of phenols in the plants (Wardrop, 1981; Wang *et al*, 2003). This was seen only in one treatment of low auxin that showed this irrespective of boron concentration. There was a low level of phenol in only one treatment of high boron and high auxin (Table. 7.6). There was no appreciable change in the phenol content of rest of the treatments. There are studies that suggest low boron can lead to lower lignin levels in the cell walls (Wardrop, 1981). Dutta and McIlrath (1964), have warned about interpretations from anatomical studies with stains as they are not specific. They mentioned about the study by Odhnoff (1961) who used quantitative analysis and demonstrated higher lignin content in boron deficient bean roots compared to normal. They have also referred to another study by Koblitz (1955), who observed increased lignin in increased levels of boron. In their own study, they reported reduced peroxidase activity with lower boron; hence concluded that there was reduced lignin. As seen in the current study there were conflicting results observed with safranin and epilofluorescence microscopy, hence, the quantitative study was also performed on the cultured wood.

Carbohydrate products	**0.03 mM auxin**		**0.3 mM auxin**		**3 mM auxin**	
Boron levels	**1 µM**	**100 µM**	**1 µM**	**100 µM**	**1 µM**	**100 µM**
4-hydroxy-5,6-dihydro-(2H)-pyran-2-one	Low	Low	Low	Low	High	High
HMF	High	High	No change	High	High	High
4-allyl phenol	High	High	Low	High	Low	Low
anhydro gluco pyranose	Low	Low	Low	High	Low	Low

Table. 7.7 *Summary of the changes observed in the carbohydrate products identified by the Py-GC-MS analysis of the cultured wood (details of measurements in Appendix 5).*

There were differences observed in the composition of carbohydrate products identified by Py-GC-MS analysis of the cultured wood compared to the control. 4-hydroxy-5,6-dihydro-(2H)-pyran-2-one was lower in the cultured wood irrespective of boron levels when the auxin concentration was 0.03 mM and 0.3 mM, while it was high when the auxin concentration was 3 mM (Table. 7.7). When the cultured wood was grown on only altered auxin concentrations in the previous study (Table. 6.9) the 4-hydroxy-5, 6-dihydro-(2H)-pyran-2-one levels in low auxin concentrations was high, whereas in high it was low. Similarly the anhydro gluco pyranose was low in most of the cultured wood grown in the present study were the boron and auxin levels were altered, whereas it was high in the previous study for both high and low concentrations of auxin (Table. 6.9). The polysaccharide composition in the cell wall was not strongly affected by boron deficiency (Noguchi *et al.*, 2003) and hence, these changes could possibly be more due to the response of the wood to the changes in auxin concentration.

7.4 The relationship between auxin and boron and lignin

There are implications of boron promoting IAA mediated processes (Birnbaum *et al.*, 1977). In a number of species, there is an increase in the endogenous levels of IAA associated with boron deficiency (Coke & Whittington, 1968; Rajarathnam *et al*., 1971).

Therefore, it is quite possible that in the current study the cultured wood cells would have higher levels of auxin under boron deficient conditions and added auxin. The high auxin levels could possibly be taking over the cellular activities and causing most of the changes during the process of xylogenesis. Hence, it is possible that the effects that were seen in the present study could be more due to not only addition of exogenous auxin but also due to increase in induced auxin levels in low boron condition (discussed in section 7.5.1)

The evidence so far strongly suggests that boron plays some essential part in the regulation of membrane function in higher plants (Pollard *et al*., 1977; Takano *et al*., 2002). Boron is known to be essential for cell wall structure and function through its role as a stabilizer of the cell wall pectic network and subsequent regulation of cell wall pore size (Brown *et al*., 2003). IAA has also been reported to inhibit basal ion uptake, especially in plants with excess boron. High IAA levels and boron deficiency have qualitatively similar effects on the membrane capacity for ion transport.

According to Lewis (1980), boron is directly involved in biosynthesis of lignin. Both p-coumarate and ferulate occupy comparable branch points in the biosynthesis of the final lignin precursors. Both can either be directly reduced to the two lignin precursors or hydroxylated or methylated, in both cases; the hydroxylated products are O-diphenols-caffeate and 5-hydroxyferulated (Lewis, 1980). It is therefore possible that complexing of borate with these intermediates is involved in metabolic control at these branch points and the primary role of boron concerns the conversion of p-coumarate to the two methoxylated lignin precursors. Boron is involved in the control of biosynthesis of lignin precursors and possibly in their polymerization as well (Lewis, 1980). Thus, in boron deficient conditions there will be an accumulation of lignin precursors. The accumulation of the coniferyl alcohol and phenols was seen only in one case where the treatment was 1 µM and 0.03 mM auxin. Hence, the same relationship exits between boron and lignin can not be made confidently in the cultured treatment study.

An attempt is made to simplify the relationship between auxin, boron and lignin in Figure. 7.7. Although the details of the interrelationship outlined in the figure have not been fully described or interpreted it is clear that boron, lignin and differentiation of

conducting tissue are intimately related and probably under-control of auxin (Lewis, 1980). There is likelihood of another scenario that could be taking place in the cultured wood called 'hyperauxiny' that is being discussed briefly in the following section.

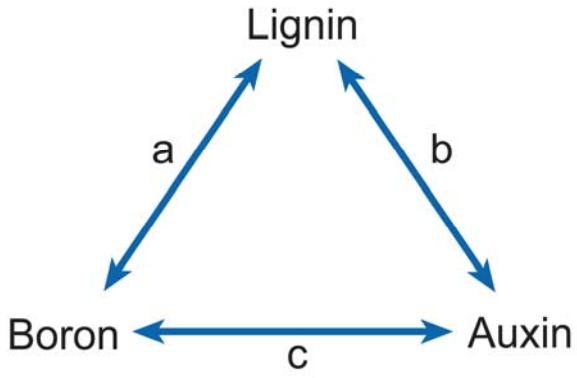

Figure. 7.7 *Interaction between auxin, boron and lignin (adapted from Lewis, 1980). (a) Boron is possibly involved in control of biosynthesis of lignin precursors and polymerisation. (b) Auxin precursors derived from autolysing tissue undergoing lignification; auxin is also involved in cellular differentiation during the process of xylogenesis. In this way lignification, cell autolysis, differentiations of xylem and auxin biosynthesis are involved in a complex regulatory interaction. (c) Auxin concentration increased in boron deficient tissues. IAA oxidase activity increases in boron deficient tissues. Phenols accumulate as a result of boron deficiency. Cell death may result with concomitant synthesis of auxin. IAA oxidase activity could be inhibited by O-diphenols.*

7.4.1 Hyperauxiny

Boron deficiency is equivalent to the state of IAA toxicity (Coke & Whittington, 1968; Lewis, 1980). Such a condition was referred to as 'Hyperauxiny' (Lewis, 1980). This can be attributed to several factors and some of them have been discussed here briefly.

In a study conducted on roots of *Vicia faba* the gross morphological effects of boron deficiency were found to be similar to those produced by high concentrations of IAA and by auxin herbicides (Coke & Whittington, 1968). Some of these effects of the 'hyperauxiny' could be seen in the cultured wood grown on low boron and high auxin (in

this section refers to only 3 mM auxin concentration). In the present study developing cells growing on low boron and high auxin showed a decrease in the number of cells compared to control by 17% on an average. The number of cells undergoing secondary wall formation also decreased under similar conditions. Thus, the cultured wood shows decrease in growth with hyperauxiny.

Coke & Whittington (1968) suggested that IAA and a cofactor could form an active complex of 'IAA oxidase'. Meanwhile, borate might complex with a diphenol inhibitor otherwise capable of complexing with oxidase at the site normally occupied by IAA co-factor. Boron deficiency could therefore lead to the accumulation of IAA because the presence of inhibitor enzyme would prevent the formation of active complex. The observation that roots grew more in period following treatment with low IAA would be explained by assuming that there was insufficient boron at the low levels to complex with the 'IAA oxidase' inhibitor and thus more time might be required to metabolise the excessive quantity of IAA supplied. Therefore, in the culture, conditions with high auxin concentration and low boron the auxin concentration would increase even further and the xylogenesis processes would be further affected. This could be one of the reasons for the decrease in the cell lumen area when the auxin concentration was high and the boron concentration was low.

According to Lewis (1980), boron deficiency leads to poor overall lignification and differentiation of xylem. Concomitant with this anomaly is the accumulation either of lignin precursors themselves or of compounds chemically or metabolically related to them. It is possible that endogenous accumulations of phenolic compounds inhibits uptake of ions in cultured cells (Lewis, 1980). This could affect the membrane permeability as pointed out by Pollard *et al.* (1977). Boron has protective effect on the membrane constituents by complex phenolic, so that oxidation of phenolics to highly toxic quinines and oxygen free radicals is prevented or limited (Cakmak *et al*., 1995). This accumulation of phenols can lead to cell death, which in turn can trigger concomitant synthesis of auxin (Lewis, 1980). Hence, in the cultured wood with low boron there would be lower lignification reducing the formation of lignin precursors, at the same time induce auxin synthesis leading to excess auxin that will further aggravate the boron deficiency condition. In the cultured wood as per the results from the Klason

lignin assay the total lignin content in the cultured wood under low boron and high auxin was even lower than control. Similarly, in the cultured wood grown in the low boron condition as per the Py-GC-MS analysis there was lower content of coniferyl alcohols (lignin precursors) and increase in coniferaldehyde, however, there was no change in the phenolic content compared to the control. Hence, it seems there is a possibility of interrelationship between the three factors and their influence on the xylogenesis in radiata pine.

7.4 Summary

The results showed much variation both between and within culture treatments. However, on an average there were some pointers that helped in understanding the role of auxin and boron on xylogenesis. Boron plays an important role in cellular activities during xylogenesis in radiata pine. It can affect the cell wall integrity, lignification and in some ways affect auxin levels in the cells. Although auxin seems to have influence on cellular division and differentiation, lignification, its activities can be influenced by the boron levels. Hence, it seems that for xylogenesis in radiata pine to occur there is interplay between boron, auxin and they, in turn can affect lignin level in the cell wall and finally the wood quality.

Chapter Eight

Summary and conclusions

8.1 Introduction

The overall aim of the thesis as discussed in chapter one was to gain an insight into intra-ring checking, and the process of xylogenesis in radiata pine. The project discussed in this thesis was a part of a larger initiative that has been undertaken in New Zealand to understand the wood quality flaw called intra-ring checking. This wood quality flaw leads to huge economic losses. Hence, a matter of grave concern to the forest industry. An ongoing collaborative effort is still underway to tackle some of the wood quality issues in radiata pine and this project happens to be a part of this campaign. The studies are being conducted with a vision of improving radiata pine wood quality and hoping to grow trees in future that will yield timber with minimum flaw and maximum profit.

8.2 Where does the check take place?

The first question that was addressed by this project was where the check occurs in radiata pine wood. Chapter two highlighted the studies that were carried out in order to address this question. The microscopy observations of the checks were analysed and inferred with the help of the fracture studies (Cote & Hanna, 1983; Donaldson, 1995, 1996, and 1997)) that were helpful in understanding the splits that occur when a check is formed in wood. Scanning electron microscopy images provided the most information about the check and it was found that checking in radiata pine wood was mostly an intercell failure that usually occurs at the cml/S_1 boundary. This study was as such a comprehensive attempt in determining the location of check in radiata pine such a study

had not been taken before. This led to the next question as to what causes a wood to check.

8.3 What properties of wood make it susceptible to checking?

After locating the site of check in the wood, the next study was conducted with an aim to find out the properties of the tracheids that made it susceptible to checking during drying. It was seen that usually the earlywood region of the wood developed checks, and the checks were widest in this region. Hence, further investigations were carried out to determine the characteristics of the earlywood that checked. A comparative study was carried out to determine if there were any inherent differences in the tracheids of the checked wood compared to non-checked wood that made it susceptible checking.

It was found that the tracheids in checked wood were different with respect to size and shape when compared to non-checked wood. The cells in checked wood were larger in the radial dimension, and had a larger lumen area and thinner radial walls (sections 3.3.5; 3.3.6; 3.3.7). In the checked wood there were cells that were found collapsed both along the check and away from the check. This was an indicator that the cells in checked wood were undergoing either shrinkage, collapse or both shrinkage and collapse (section 3.3.2.1 and section 3.3.3). The net movement that is produced in the wood during drying (Booker & Sell, 1998; Pang, 2002) could lead to shearing and splitting amongst the cell wall layers (Pang, 2002), increasing the likelihood of the formation of check. The larger diameter tracheids were found to be more vulnerable to cavitations (Wang *et al.*, 2003), and thicker walls can serve as protection against it (Wang & Aitken, 2001). Hence, it seems that cells that were larger in size, as well as having thinner cell walls were not well equipped to withstand the differential stresses generated during drying of wood and may be prone to develop checks.

Light microscopy that was used for this study revealed differential staining in checked and non-checked wood with safranin-fast green stain. Hence, further investigations were carried out to determine if there were any differences in the lignin levels in the cell wall. As the checking takes place at the cml/S_1 boundary study was also carried out to look at the other important component of the compound middle lamella that is pectin (Buchannan *et al*., 2000).

8.4 Does lignin influence checking in wood?

A comparative study was carried out between the checked wood and non-checked wood to determine if there were differences in the lignin levels and the lignin distribution in the cell wall layers. Qualitative studies as well as quantitative studies were carried out to study the lignification of the checked and non-checked wood. The qualitative study was carried out with the help of safranin-fast green stained slides, where the intensity of the staining of the cell wall with safranin was used as an indicator of the levels of lignin present in the cell wall. It was found that the checked wood (Figure 4.3) had lower lignin compared to the non-checked wood. However, safranin stain is not considered to be a very specific lignin (Sarkanen & Ludwig, 1971). Hence, the wood was also observed with the help of epifluorescence microscopy that is considered to be a better indicator of the presence of lignin in wood (Scott *et al*., 1969; Fergus *et al*., 1969; Fukuzawa & Imagawa, 1981; section 4.3.2). Similar observations to the safranin stain were made, the checked wood displayed lower levels of lignin compared to the non-checked wood. Ultrathin sections stained with potassium permanganate were also used to detect lignin at the ultrastructural level in the cell wall layers with the help of TEM. It was found that there was lower lignin staining in the compound middle lamella and S_1 layers, of the checked wood compared to the non-checked wood. It was also observed that the non-checked wood had a more homogenous lignification overall across the wood (Putoczki, 2006). Two biochemical assays, the Klason lignin method and the acetyl bromide assay were carried out to measure the lignin content in the checked and non-checked wood. However, the results were not significant (Putoczki, 2006). Hence, it seems that it is not the total lignin content but the distribution of the lignin in the cell wall that could play a more crucial role in checking (Jackson *et al.*, 2004). The results from the previous fracture studies and the current study indicate that lignification of the cml and the S_1 layers play an important role in the integrity of the cell wall. The results from the current study and the earlier observations of lignin in the checked wood (Donaldson & Singh, 2002; Donaldson, 2002) have a consensus that lower lignin levels could make the wood susceptible to checking.

Pectin, another important component of the cml, showed no significant difference between checked and non-checked wood. If cell wall thickness and lignin were crucial

factors for checking then the presence of other structures in the wood like rays and resin canals that are usually thin walled and unlignified could serve as point of initiation. Hence, a study was carried out to understand the role of other structures in wood.

8.5 Do structural flaws lead to weak sites that can initiate checking?

Radiata pine wood has a simple structure with the bulk of the wood being composed of tracheids (section 1.4). However, there are other structures present in radiata pine wood such as ray and resin canals that pose a possibility of being the weak links that can initiate checking.

Observations of the checked surface showed that rays and resin canals were associated with checks. However, the resin canals were seen only in 10% of the total 60 cases of checked surface observed. Every ray comes in contact with tracheid (Esau, 1967). However, not all of them check. From this analysis it was ruled out that rays or resin canals were the main cause of checking. However, on further analysis it was seen that the checked wood had more ray tissue exposed along the checked surface compared to non-checked wood. Hence, there could be a possibility that if more rays occupied area in the wood that could make a difference in the wood structure and make it vulnerable to check (section 5.3.1.1). Resin canals were not directly involved in checking. However, their scattered arrangement in the checked wood was an indication of possible disturbances during the development of the wood (section 5.3.2.1).

Another characteristic that was found in the checked wood tracheids was that they had larger pit compared to the non-checked wood. Pits could play an important role in checking in radiata pine. During the process of kiln drying water is lost from wood and the pits aspirate. According to Booker (2004) there is a differential contraction between earlywood and latewood as the water leaves the cells and cell walls and this can lead to formation of check. If the wood has bigger pits, there is the possibility of water leaving the cells at a faster rate. According to Simpson *et al.,* (2002), faster drying of wood can cause checking. In addition, Pang (2002) also pointed out that pits could influence shrinkage and check formation. Hence, it seems that the larger pits in the checked wood could contribute to checking in radiata pine.

The anatomical, ultrastructural and chemical composition of wood influences its mechanical properties. Hence, investigations were carried out to study some of the important mechanical properties of wood such as density, microfibril angle and modulus of elasticity.

8.6 Do mechanical properties influence checking in wood?

A study was conducted to understand the influence of the mechanical properties of wood on checking in radiata pine (detailed report in Appendix 7). Density, microfibril angle (MFA) and modulus of elasticity (MOE) of checked wood and non-checked wood were measured and compared using various techniques such as SilviScan-2, X-ray diffraction and modified Fullam's micro-test stage.

It was found that the checked wood had a lower density compared to the non-checked wood. Low density in wood could primarily be an expression of thin cell walls and possibly large lumen area(section 3.3.5, 3.3.6) and in the current study it was that the checked wood had thinner walls and larger lumen (chapter three). Overall as per the X-ray diffraction measurements it was seen that the checked wood had a tendency towards higher MFA as compared to non-checked wood (there were only five samples that were analysed for this study due to limitations of the resources). Higher MFA means that there will be more shrinkage in the wood (Huang *et al*., 2003), this can possibly lead to checking in wood. The MOE is one the most important mechanical properties of solid timber and is the composite function of MFA and density (Evans & Ilic, 2001; Lindstrom *et al*., 2002; 2004). There were variations in the data, though there was weak trend on analysis with the measurements obtained from one of the techniques (modified Fullam's micro-test stage) that showed checked wood had lower MOE compared to non-checked wood.

8.7 Is organ culture suitable to study xylogenesis in *Pinus radiata* ?

There are many techniques in use to study the process of xylogenesis in plants (section 1.9, chapter 1). However, the organ culture study was determined to be the tool of choice for this study. There are many advantages to this technique (section 1.10), the most important one being that the results obtained from the study would be relevant to the mechanisms that take place in the same species. Hence, the study would have more relevance and application in the real world. Unlike the criticism for the model plant study where it is a view point that they can not represent all the life forms (Chaffey, 2002a; Przemyslaw, 2004). This study was an integrated part of the broader study where an attempt was being made to understand the process of xylogenesis in radiata pine. Hence, using organ culture system would be the closest way to mimic the mechanism of the tree and study the changes it undergoes as the conditions were altered. The organ culture was ideal as it was easy to control the conditions of the experiment and monitor the progress of xylogenesis with ease without any interference from external conditions and the whole plant (section 1.10).

8.8 Did the organ culture respond to the culture conditions and were there changes in the process of xylogenesis observed?

The results and analysis presented in chapter 6 and 7 clearly indicate that there was successful initiation of the culture and there were changes in the wood with respect to the culture conditions. Hence, the organ cultures did respond to the treatments. There was successful uptake of the auxin and boron by the organ cultures from the medium. There were a number of changes observed with respect to wood properties in the cultured wood and some of the changes observed were in a dosage dependent manner (like colour of wood, cell increase, cell expansion). The organ culture was successful as far as wood formation in a controlled, monitored condition is concerned. However, there was much variation observed and a lack of clear trends with respect to some of the wood properties. Generally, the same clonal material from the same site was used in order to reduce variation. However, trees from identical trees can produce wood with varying properties

(Mellerowicz, 2001). Kumar and team (2004) conducted a study on the influence of genotype on wood formation in radiata pine and found large tree-to-tree variation at each site. Similar results have been reported in field trials of radiata pine (Beets *et al*., 2003) as well as in another concurrent study conducted in radiata pine organ cultures where much variability in the data was reported (Putoczki, 2006).

Additional trial studies were conducted to see the impact of auxin transport in the organ cultures. This was conducted by changing the polarities in the organ cultures by growing them in various ways (Appendix 6). However, it seemed that the organ cultures grew as long as there was a diffusion gradient. The cultured wood did not show any abnormality either externally or in the process of xylogenesis even when grown upside down (details of the study and measurements in Appendix 6). Hence, there was successful modification and development of the organ cultures of radiata pine wood from trees that were at least ten years old. This was significant as most studies have used a culture system from much younger tissue (Savidge, 1993; Wodzicki, 2001). In the present study older tissue system from trees harvested straight from radiata pine plantations has been used.

8.8.1 Response of the cultured wood grown on varying auxin concentrations

Generally, experiments with exogenous auxin have yielded the same result as in the current study (section 6.4). Though there was much variation in the data there were pointers that could be used to make inferences about the role of auxin in xylogenesis. Overall, there was an increase in cellular division and expansion in the cultured wood in the presence of exogenous auxin. These results find support in the previous studies where it was seen that exogenous auxin controlled cell division, cell expansion and differentiation in plant tissues (Zajaczkowski, 1973, Petrášek *et al*., 2002). The existing cells also responded by changing their cell wall thickness. On the whole mostly there was an increase in cell wall thickness with increase in auxin. One of possible explanation for the increase in thickness was the increase in lignification in the cell walls due to the presence of exogenous auxin. According to the results of microscopy this seemed true. However, the data from the biochemical assays was not as conclusive.

8.8.2 Response of the cultured wood grown on varying auxin and boron concentrations

There seemed to be interplay between the auxin and boron levels on the process of xylogenesis. It is possible that boron could be mediating auxin related processes (Birnbaum *et al*., 1977). The interpretation of the data was done with much caution as there was variability in the data both 'between trees' and 'within cultures'. There were no distinct trends that emerged from the data analysed.

Some changes seen in the cultured wood gave an indication of possible interactions between boron, auxin and their role in xylogenesis. The developing cells seemed to be most affected by boron deficient conditions. There was less cellular activity such as cell division and expansion compared to high boron conditions irrespective of auxin concentrations. When the auxin concentration was high (3 mM) and boron concentration was low (1µM) the changes in the cells undergoing development were more pronounced. A decrease in the cell lumen area and a reduction in number of cells were observed. These changes that were observed were attributed to 'hyperauxiny'. Hyperauxiny effects growth and is a condition that results in boron deficient tissues that have either low auxin concentration or the auxin concentration is in excess, the gross morphological effect is like that of boron deficiency (Coke& Whittington, 1968; section 7.5). Boron and auxin can also affect lignification, in the present organ culture study there were conflicting and variable results, hence, a definite pattern of interaction between lignin, boron and auxin could not be established.

8.9 How do these results relate to checking in radiata pine?

The study of the checked wood led to the suggestion that the tracheid size, shape and thickness of the wall, along with lignin content, could play an important in checking (chapter three and four). The auxin and boron concentrations were altered to understand their impact on xylogenesis. Auxin increased cellular activity and led to changes in the cell size, so it is likely that high auxin levels in radiata pine can lead to changes in the tracheid geometry. In the organ cultures with altered boron and auxin levels it was found that boron levels could influence the developing cells irrespective of the auxin levels.

One important observation was that the cml was swollen when grown on boron deficient conditions irrespective of the auxin concentrations. A similar response of the cml in boron deficient conditions has been reported earlier (Ishii *et al.*, 2001; Putoczki, 2006). There have been reports of decreased cell wall resistance to mechanical stress, weakening of wall to wall contacts between cells and weakened cml in boron deficiency (Fleischer *et al*., 1998). Hence, boron deficiency could lead to weakness in the cell wall that could prove detrimental to the wood quality. Furthermore, an increase in auxin could further exaggerate the impact of boron deficiency (Coke& Whittington, 1968; Lewis, 1980; section 7.5). Hence, if fast growing radiata pine trees grow on boron deficient soil it is possible that xylogenesis will be affected, more importantly the cell wall formed could be weaker and vulnerable to checking (Figure. 8.1).

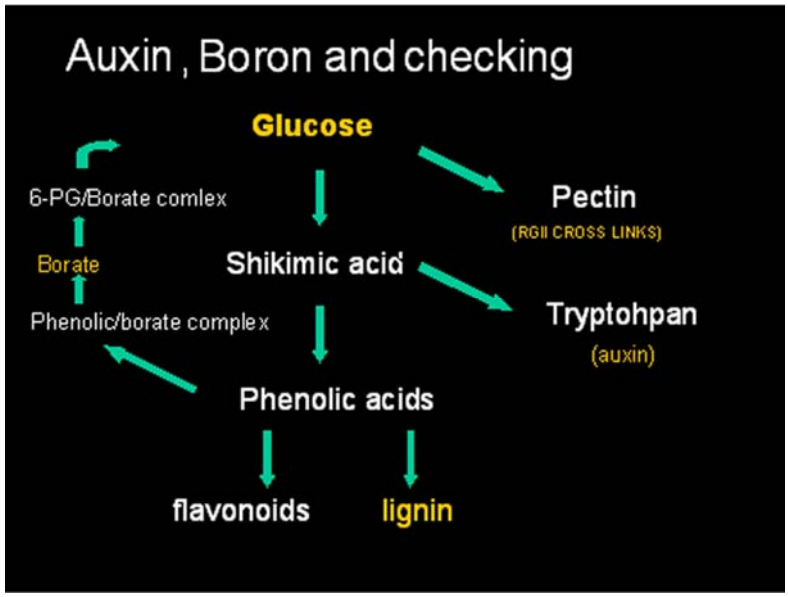

Figure. 8.1 *Presents a flow chart that is an attempt to try and simplify the complex inter-relationship between auxin, boron, lignin and pectin, amongst many other factors that could affect intra-checking. These factors play an important role in xylogenesis and integrity of the cell wall and in turn influence checking.*

8.10 Conclusion

The location of the check was determined, and a range of microscopy techniques have been used to characterise the anatomy of the radiata pine wood. There is now greater understanding about some of the properties of wood that can lead to checking. Furthermore, there was successful establishment of organ culture of the radiata pine that provides a beneficial system where trials can be carried out in a controlled environment and the results can be obtained within short period of time. The advantage of the quick turn around time is that the results obtained from the studies can be applied in the fields. However, there was much variation in the data observed in the present organ culture study and another concurrent study (Putoczki, 2006). Future work has to be designed and interpreted with caution. The project has added more to the knowledge about intra-ring checking and some of the processes of xylogenesis in radiata pine.

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Appendix One

Supplemental comparative analysis of the checked and non-checked wood

This appendix presents the details of the data presented in Tables 3.1 and 3.2 in chapter three.

Table 1 (Expanded version of Table 3.1). Wood samples that underwent intra-ring checking upon oven drying had differences in their fibre and lumen width and shape as well as cell wall thickness and lignin distribution in growth ring 7. Kruskal-Wallis non-parametric statistic was used to detect differences between the medians of the fibre characteristics in the severe, moderate and no checking groups. Groups indicated by colour, when there was no difference between samples they were given the same color. Key: ▢ Indicates the largest sample, ▢ the intermediate, and ▢ the smallest.

Wood characteristic	**Severe checking**	**Moderate checking**	**No checking**
fibre cross sectional area			
Earlywood			
Median (□m^2)	1134.6	1166.3	903.66
Mean rank with Groupings (P=0.0006, groupings □=0.001 crit. Z=3.59)	2369.6	2454.1	1722.3
Average ± S.E.M.	1195.7 ± 12.5	1229.3 ±17.5	918.0 ± 11.1
Sample size	2035	1035	1319
Latewood			
Median (□m^2)	847.34	888.73	709.35
Mean rank with Groupings (P=0.0012 groupings □=0.001 crit. Z=3.59)	3247.7	3550.8	2508.6
Average ± S.E.M.	853 .4± 7.1	940.4 ± 10.2	710.6 ±6.4
Sample size	2555	1600	2014
Fibre width in radial axis			
Earlywood			
Median (□m)	43.15	43.15	36.02
Mean rank with Groupings (P=0.0017 groupings □=0.001 crit. Z=3.59)	2477.0	2489.8	1528.6
Average ± S.E.M.	43.40±0.22	43.59 ±0.31	36.33±0.22
Sample size	2035	1035	1319
Latewood			
Median (□m)	31.90	35.65	28.14
Mean rank with Groupings (P=0.0028 groupings □=0.001 crit. Z=3.59)	3201.4	3984.1	2223.1
Average ± S.E.M.	32.22±0.14	35.89±0.20	28.53±0.13
Sample size	2555	1600	2014

Wood characteristic	Severe checking	Moderate checking	No checking
Fibre width in tangential axis			
Earlywood			
Median (μm)	35.65	36.40	33.40
Mean rank with Groupings (P<0.0001 α=0.001 crit. Z=3.59)	2280.5	2366.7	1928.4
Average ± S.E.M.	35.61±0.23	36.38 ± 0.31	32.83 ±0.25
Sample size	2035	1035	1319
Latewood			
Median (μm)	33.40	34.15	32.64
Mean rank with Groupings (rank P<0.0001 α=0.007 crit. Z=3.04)	3155.3	3329.9	2801.3
Average ± S.E.M.	33.11±0.18	34.25 ±0.22	31.21 ±0.17
Sample size	2555	1600	2014
Fibre width ratio (radial/tangential)			
Earlywood			
Median	1.22	1.20	1.10
Mean rank with Groupings (P<0.0006 α=0.001 crit. Z=3.59)	2369.6	2454.1	1722.3
Average ± S.E.M.	1.28±0.007	1.24±0.009	1.16±0.008
Sample size	2035	1035	1319
Latewood			
Median	0.98	1.06	0.91
Mean rank with Groupings (rank P<0.0006 α=0.007 crit. Z=3.04)	3089.8	3645.2	2633.8
Average ± S.E.M.	1.03±0.006	1.09 ±0.006	0.96±0.005
Sample size	2555	1600	2014
Lumen cross sectional area			
Earlywood			
Median (μm^2)	754.27	859.02	622.41
Mean rank with Groupings (P=0.0006 α=0.001 crit. Z=3.59)	2310.7	2591.7	1786.6
Average ± S.E.M	832.42±10.53	921.24±13.64	645.06±7.81
Sample size	1825	1113	1474
Latewood			
Median (μm^2)	430.85	460.42	365.10
Mean rank with Groupings (P=0.0006 α=0.001 crit. Z=3.59)	3235.8	3519.4	2685.6
Average ± S.E.M.	457.83+4.50	514.43+7.08	384.21+4.05
Sample size	2613	1603	2041

continued

Wood characteristic	Severe checking	Moderate checking	No checking
Lumen width in radial axis			
Earlywood			
Median (μm)	36.77	37.90	30.39
Mean rank with Groupings (P=0.0018 α=0.03 crit. Z=2.58)	2487.6	2617.9	1547.9
Average ± S.E.M.	37.12±0.22	38.10±0.27	30.59±019
Sample size	1825	1113	1474
Latewood			
Median (μm)	23.64	25.52	20.64
Mean rank with Groupings (P=0.0017 α=0.001 crit. Z=3.59)	3316.1	3816.3	2349.7
Average ± S.E.M.	24.16+0.12	26.33+0.18	20.90+0.12
Sample size	2613	1603	2041
Lumen width in tangential axis			
Earlywood			
Median (μm)	27.77	29.64	26.64
Mean rank with Groupings (P<0.0001 α=0.001 crit. Z=3.59)	2217.9	2476.1	1988.8
Average ± S.E.M.	27.86±0.22	29.71±0.26	26.13±0.20
Sample size	1825	1113	1474
Latewood			
Median (μm)	22.89	23.26	22.51
Mean rank with Groupings (P<0.0001 α=0.02 crit. Z=2.71)	3151.4	3313.1	2955.7
Average ± S.E.M.	23.00±0.14	23.87±0.20	22.06±0.14
Sample size	2613	1603	2041
Lumen width ratio (radial/tangential)			
Earlywood			
Median	1.37	1.28	1.17
Mean rank with Groupings (P<0.0006 α=0.001 crit. Z=3.59)	2474.0	2254.4	1839.2
Average ± S.E.M.	1.44±0.01	1.34±0.01	1.24±0.01
Sample size	1825	1113	1474
Latewood			
Median	1.07	1.12	0.96
Mean rank with Groupings (P=0.0006 α=0.001 crit. Z=3.59)	3282.7	3595.7	2565.7
Average ± S.E.M.	1.12±0.006	1.18±0.009	1.00±0.006
Sample size	2613	1603	2041

continued

Wood characteristic	Severe checking	Moderate checking	No checking
Cell wall thicknes in radial axis			
Earlywood			
Median (μm)	2.70	2.54	2.89
Mean rank with Groupings (P<0.0001 α=0.03 crit. Z=2.58)	287.8	251.1	332.7
Average ± S.E.M.	2.73±0.03	2.64±0.04	2.99±0.06
Sample size	200	190	190
Latewood			
Median (μm)	5.33	5.70	5.11
Mean rank with Groupings (P<0.0001 α=0.05 crit. Z=2.39)	281.4	338.7	251.8
Average ± S.E.M.	5.42+0.08	5.83+0.09	5.07+0.11
Sample size	200	190	190
Cell wall thickness in tangential axis			
Median (μm)	2.56	2.12	2.53
Mean rank with Groupings (P<0.0001 α=0.05 crit. Z=2.39)	350.3	187.0	331.0
Average ± S.E.M.	2.70±0.04	2.15±0.03	2.70±0.06
Sample size	200	190	190
Latewood			
Median (μm)	5.48	6.27	6.22
Mean rank with Groupings (P<0.0001 α=0.0001 crit. Z=3.59)	225.5	336.6	312.8
Average ± S.E.M.	5.52+0.08	6.33+0.09	5.94+0.11
Sample size	200	190	190
Lignin proportion in cell wall			
Earlywood			
Median	0.094	0.147	0.230
Mean rank with Groupings (P=0.0001 groupings α=0.06, crit z =2.33)	23.4	33.5	48.2
Average ± S.E.M.	0.127±0.02	0.161±0.01	0.243±0.02
Sample size	28	20	17
Latewood			
Median	0.3121	0.3718	0.6838
Mean rank with Groupings (P=0.0130 groupings α=0.15 crit. Z =1.96)	26	20.5	36.4
Average ± S.E.M.	0.420+0.06	0.346+0.04	0.590+0.06
Sample size	22	17	14

continued

Wood characteristic	Severe checking	Moderate checking	No checking
Middle lamella width in radial axis			
Earlywood			
Median (μm)	0.95	0.96	0.96
Mean rank with Groupings (P=0.7695)	285.2	289.2	297.3
Average ± S.E.M.	0.97±0.02	0.97±0.02	0.99±0.02
Sample size	200	190	190
Latewood			
Median (μm)	1.14	1.14	0.87
Mean rank with Groupings (P<0.0001 groupings α=0.001 crit. Z=3.59)	320.0	324.8	225.2
Average ± S.E.M.	1.13±0.03	1.13±0.02	0.92±0.02
Sample size	200	190	190
Middle lamella width in tangential axis			
Earlywood			
Median (μm)	1.41	1.33	1.43
Mean rank with Groupings (P=0.0113 groupings α=0.01 crit. Z=2.94*)	292.3	263.9	315.2
Average ± S.E.M.	1.43±0.02	1.39±0.02	1.49±0.03
Sample size	200	190	190
Latewood			
Median (μm)	1.88	2.09	1.45
Mean rank with Groupings (P<0.0001 groupings α=0.002 crit. Z= 3.40)	285.1	360.7	225.9
Average ± S.E.M.	1.88±0.03	2.14±0.03	1.65±0.05
Sample size	200	190	190

*mixed groupings –severe and no check formed on group, severe and moderate a second group.

Table 2 (Expanded version of Table 3.2). Fibre characteristics as defined by FibreLab™. Earlywood and latewood samples were dissected from growth ring 7 and macerated. Fibres from samples that underwent intra-ring checking upon oven drying had differences in their cells, cell walls and lumens as reported below. Kruskal-Walis statistic was used to detect differences between the groups because data were not normally distributed. Groups are indicated by colour. When there was no difference between samples they were given the same colour.

Note cell wall thickness measurements are unrealistically high. They were roughly 5.5 times higher than those measured using image analysis. The trends for changes in cell wall width are opposite to those measured with image analysis (compare with Table 2). Furthermore, the difference in call thickness between earlywood and latewood was not detected. This disparity between image analysis and FibreLab™ cell wall thickness has been reported by others (Richardson et al 2003). Accurate cell wall thickness measurements are beyond the limit of resolution for this machine.This means that **fibre cross sectional area** and **fibre volume** which are calculated using **cell wall thickness** are also **dubious values!**

Key: indicates the largest sample, the intermediate, and the smallest.

Wood characteristic	**Severe checking**	**Moderate checking**	**No checking**
fibre length			
Earlywood			
Median (mm)	2.74	2.74	2.72
Mean rank with Groupings (P=0.1707)	5194.0	5324.4	5213.3
Average ± S.E.M.	2.68±0.01	2.73±0.01	2.69±0.01
Sample size	3016	3233	4234
Latewood			
Median(mm)	2.97	2.98	2.52
Mean rank with Groupings (P=0.0018 groupings α=0.001 crit. Z=3.59)	4630.8	4699.8	3350.8
Average ± S.E.M.	2.94±0.01	2.96±0.02	2.52±0.01
Sample size	3144	1831	3296

continued

Wood characteristic	Severe checking	Moderate checking	No checking
Fibre width			
Earlywood			
Median (μm)	49.06	49.80	46.53
Mean rank with Groupings (P=0.0006 groupings α=0.001 crit. Z=3.59)	5443.8	5750.9	4709.7
Average ± S.E.M.	49.07±0.21	50.56±0.20	46.97±0.13
Sample size	3016	3233	4234
Latewood			
Median (μm)	47.97	47.11	42.51
Mean rank with Groupings (rank P=0.0018 groupings α=0.04 crit. Z=2.47)	4733.4	4554.9	3333.4
Average ± S.E.M.	48.60±0.16	48.29±0.22	43.60±0.16
Sample size	3144	1831	3296
fibre cross sectional area (*based on cell wall thickness)			
Earlywood			
Median (μm^2)	1512.8	1543.8	1362.4
Mean rank with Groupings (P=0.0006,groupings α=0.004 crit. Z=3.21)	5447.9	5720.6	4729.9
Average ± S.E.M.	1582.2±13.15	1663.8±12.90	1428.7±8.66
Sample size	3016	3233	4234
Latewood			
Median (μm^2)	1481.7	1451.5	1169.7
Mean rank with Groupings (P=0.0018 groupings α=0.001 crit. Z=3.59)	4699.8	4628.4	3324.6
Average ± S.E.M.	1562.1±10.80	1575.1±15.26	1267.9±9.95
Sample size	3144	1831	3296
fibre volume (*based on cell wall thickness)			
Earlywood			
Median (mm^3)	4.01	4.17	3.70
Mean rank with Groupings (P<0.0001,groupings α=0.001 crit. Z=3.59)	5336.9	5623.5	4883.1
Average ± S.E.M.	4.33±0.05	4.70±0.05	3.95±0.33
Sample size	3016	3233	4234
Latewood			
Median (mm^3)	4.25	4.19	2.94
Mean rank with Groupings (P=0.0029 groupings α=0.001 crit. Z=3.59)	4788.2	4754.8	3170.1
Average ± S.E.M.	4.68±0.05	4.82±0.07	3.26±0.03
Sample size	3144	1831	3296

continued

Wood characteristic	Severe checking	Moderate checking	No checking
fibre curl			
Earlywood			
Median	3.0	3.0	3.0
Mean rank with Groupings (P=0.013),groupings α=0.06 crit. Z=2.33)	5164.6	517.7	5346.2
Average $\pm$ S.E.M.	5.47$\pm$0.16	5.35$\pm$0.16	6.73$\pm$0.17
Sample size	3016	3233	4234
Latewood			
Median	3.0	3.0	3.0
Mean rank with Groupings (P<0.0001 groupings α=0.001 crit. Z=3.59)	3930.3	3987.4	4414.7
Average $\pm$ S.E.M.	4.19$\pm$0.12	4.21$\pm$0.15	6.89$\pm$0.20
Sample size	3144	1831	3296
Cell wall thickness *			
Earlywood			
Median (μm)	13.76	13.76	12.9
Mean rank with Groupings (P<0.0001,groupings α=0.001 crit. Z=3.59)	5442.3	5610.3	4818.1
Average $\pm$ S.E.M.	13.61+0.07	13.89+0.06	13.1+0.05
Sample size	3016	3233	4234
Latewood			
Median (μm^2)	13.76	13.76	12.04
Mean rank with Groupings (P=0.0018 groupings α=0.001 crit. Z=3.59)	4582.0	4723.0	3384.5
Average $\pm$ S.E.M.	14.02+0.06	14.32+0.07	12.61+0.06
Sample size	3144	1831	3296

*dubious values as cell wall thickness measurements are beyond the limit of resolution for FibreLab^{TM}.

Appendix Two

Statistics data details presented in chapter five

The details of the statistical analysis that were carried out on the data collected from ray, resin canals and pits observed in the checked and the non-checked wood.

ANOVA Tables

Nested ANOVA's were carried out using the S Plus statistical package.

Number of resin canals per unit area

Error:tree
	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
check	2	248.0462	124.0231	1.248911	0.3321401
tree	9	893.7449	99.3050		

Error: Within
	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	31	587.8833	18.96398		

Note that the level of tree did not explain a significant level of variation in the numbers of resin canals.

Number of rays per unit area

Error: tree
	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
check	2	0.08196831	0.04098415	4.493346	0.04434151
tree	9	0.08208969	0.00912108		

Error: Within
	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	25	0.03393757	0.001357503		

Size of pit aperture

Error: tree
	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
check	2	3.202564	1.601282	10.61293	c
tree	6	0.905282	0.150880		

Error: Within

```
          Df Sum of Sq      Mean Sq F Value Pr(F)
Residuals 596  4.754833 0.007977908
```

Width of pit border

```
Error: as.factor(Sample)
         Df Sum of Sq  Mean Sq  F Value       Pr(F)
   check  2  3558.965 1779.482 16.15556 0.003841311
Residuals  6   660.881  110.147

Error: Within
          Df Sum of Sq  Mean Sq F Value Pr(F)
Residuals 595  4001.313 6.724896
```

Radial width of tracheids

```
Error: tree
         Df Sum of Sq  Mean Sq  F Value       Pr(F)
   check  2  5740.103 2870.051 14.12706 0.008766203
    tree  5  1015.799  203.160

Error: Within
          Df Sum of Sq Mean Sq F Value Pr(F)
Residuals 375  7790.473 20.7746
```

UV fluorescence intensity

```
Error: tree
         Df  Sum of Sq    Mean Sq  F Value      Pr(F)
checking  2   0.05533743 0.02766871 21.16838 0.04510929
tree      2   0.00261415 0.00130708

Error: Within
         Df  Sum of Sq     Mean Sq F Value Pr(F)
Residuals 45 0.05851201 0.001300267
```

Appendix Three

Details of the preparation of the media

This appendix presents the details of the preparation f the growth media preparation as discussed in chapter six and seven

Table 3: The standard medium composition used for growth of radiata pine organ cultures. The chemicals used for the culture media were obtained from BDH (Poole, England), or Sigma Aldrich (St. Louis, USA).

Components for medium	**mg/L**	**mM**
KNO_3	475	4.70
NH_4NO_3	412	5.15
$CaCl_2.2H_2O$	110	0.75
$MgSO_4.7H_2O$	93	0.38
$MnSO_4.4\ H_2O$	6	0.026
$CuSO_4.5H_2O$	0.06	0.0002
$CoCl_2.6H_2O$	0.06	0.00025
$Na_2Mo\ O_4$	0.06	0.0003
KI	0.2	0.0012
KH_2PO_4	43	0.32
H_3BO_3	1	0.016
Na_2EDTA	9.3	0.025
$FeSO_4.7H_2O$	7	0.025
myo-inositol	25	0.139
Nicotinic acid	0.125	0.001
Pyridoxine HCl	0.125	0.0006
Thiamine HCl	0.125	0.0004
Glycine	0.5	0.0067
Sucrose	20,000	58.4
NAA	0.5	0.00268
Bacto-agar gelling agent	8,000	n/a
The pH of the media was adjusted to 5.8		

Table 4. The preparation boron free growth medium. The details of the stock solutions preparations and storage (Putoczki, 2006).

Macronutrients	Savidge mg/L	g/L	10x stock g/L
KNO_3	475	0.475	4.75
NH_4NO_3	412	0.412	4.12
$CaCl_2\ 2H_2O$	110	0.110	1.10
$MgSO_4\ 7H_2O$	93	0.093	0.93
KH_2PO_4	43	0.043	0.43

Micronutrient	Savidge mg/L	g/L	100x stock g/L
$MnSO_4$	6	0.006	0.6
$CuSO_4\ 5H_2O$	0.060	0.00006	0.006
$CoCl_2\ 6H_2O$	0.060	0.00006	0.006

Micronutrients that cannot go through the column

Micronutrient	Savidge mg/L	g/L	100x stock g/L
$NaMoO_4\ 2H_2O$	0.060	0.00006	0.006
KI	0.20	0.002	0.02
H_3BO_3	As per required concentration		

Vitamens	Savidge mg/L	g/L	100x stock g/L
myo-inositol	25	0.25	2.5
			1000x stock g/L
Nicotinic acid	0.125	0.000125	0.125
Pyridoxine HCl	0.125	0.000125	0.125
Thiamine	0.05	0.00005	0.05
Glycine	0.5	0.0005	0.05

chelators	Savidge mg/L	g/L	100x stock g/L
$FeSO_4\ 2H_2O$	7	0.007	0.7
Na_2EDTA	9.3	0.0093	0.93

$FeSO_4\ 2H_2O$ and Na_2EDTA= FeEDTA were heated slightly to dissolve.

MAKING MEDIUM FROM STOCK SOLUTIONS

	1 L	500 mL
Macronutrients	100 mL	50 mL
Micronutients (100x stock) N.B to be added to the mix that ahs been passed through the column $NaMoO_4$ $2H_2O$ KI H_3BO_3		
Vitamins		
100x stock or weigh to add myo-inositol	10 mL	5 mL or weigh
1000x stock	1 mL	0.5 mL
100x stock	10 mL	5 mL
Chelators	10 mL	5 mL
Agar	8 g	4 g

The macronutrients and micronutrient stock solutions were made in and stored in freezer. The chelators were stored at 4°C. Sucrose and myo-inositol were weighed directly into the media before passing through the column. KI and $NaMoO_4$ $2H_2O$ were added to the mixture that was passed through the media as these nutrients can bind to the media. NAA and H_3BO_3 were added as per the required concentration for the experiment.

All the solutions were made in autoclavable polypropylene containers, pH adjusted to 5.8 prior to autoclaving the media.

Appendix Four

Supplemental auxin culture analysis

The appendix presents supplemental culture analysis and statistical analysis for the data presented in chapter six.

Table 3: The details of the measurements of the three sets that were cultured from two different trees and treated with different concentrations of auxin.

Set number 1 culture date: 22nd Apr 04 Growth period: 1 month Rotorua	0.003 mM NAA	0.03 mM NAA	0.3 mM NAA	3 mM NAA	Control
Changes in cell number					
Cambial region cell number	3.67 ±1.03	5.67 ±2.16	5.2 ±0.84	4.8 ±0.45	3.83 ±1.33
RE region cell number	18.67 ±0.82	22.33 ±3.39	22.4 ±4.39	16.6 ±6.17	20.167 ±0.75
Developing cells region	16.33 ±1.033	14 ±1.27	13 ±1.22	20.8 ±1.64	13.33 ±1.03
Cambial region cells					
lumen area (μm2)	187.21 ±55.67	117.26 ±57.45	181.15 ±82.88	180.37 ±55.42	153.13 ±40.09
radial length (μm)	7.24 ±1.27	4.79 ±1.53	7.05 ±1.44	6.55 ±1.23	5.35 ±1.14
tangential length width (μm)	25.71 ±5.515	23.72995 ±5.67	25.15475 ±6.49	27.63282 ±6.98	28.93033 ±5.95
RE region cells					
lumen area (μm2)	622.39 ±170.59	568.06 ±182.73	598.31 ±271.46	620.97 ±234.22	650.027 ±204.58
radial length (μm)	33.05 ±4.51	31.27 ±4.88	34.96 ±9.71	33 ±6.35	32.54 ±4.91
tangential length width (μm)	24.96 ±4.11	24.37 ±4.26	22.47 ±6.33	24.73 ±5.64	25.66 ±4.99
percent cell wall area (%(μm^2)	15.21	18.72	41.55	43.76	43.93
Existing cells					
lumen area (μm2)	679.7439 ±298.9869	786.0649 ±362.5108	852.574 ±345.8124	888.0695 ±343.7076	1052.694 ±450.4

radial length (μm)	38.84 ±9.01	36.29 ±7.91	37.77 ±8.03	39.3 ±7.92	45.06 ±9.91
tangential length width (μm)	22.13 ±6.75	27.41 ±8.02	27.04 ±6.61	29.48 ±7.76	30.26 ±7.28
percent cell wall area (%(μm^2)	43.59	34.92	46.95	56.53	52.27
percent lignin area in CML/	19.97	15.93	36.17	18.26	12.86
Set number 2	0.003 mM NAA	0.03 mM NAA	0.3 mM NAA	3 mM NAA	Control
culture date: 26th Apr 04					
Growth period: 2 months Rotorua					
Changes in cell number					
Cambial region cell number	3.17 ±0.41	3.286 ±0.49	4.2 ±0.45	5.67 ±1.21	3.17 ±0.41
RE region cell number	19.5 ±1.517	24 ±3.92	27.2 ±3.11	24.67 ±2.5	17.5 ±1.76
Developing cells region	17.67 ±1.862	21.33 ±1.03	17.2 ±3.42	13.67 ±2.42	13.33 ±1.03
Cambial region cells					
lumen area (μm2)	198.58 ±71.78	175.25 ±43.78	194.92 ±73.69	147.90 ±49.17	168.03 ±57.09
radial length (μm)	6.59 ±1.89	7.21 ±1.19	7.06 ±2.14	6.49 ±1.69	5.89 ±1.27
tangential length width (μm)	29.69 ±4.79	24.1 ±3.31	27.12 ±5.78	22.8 ±4.51	28.19 ±6.60
RE region cells					
lumen area (μm2)	667.59 ±254.29	582.45 ±194.09	606.83 ±226.01	518.49 ±169.28	688.59 ±276.66
radial length (μm)	33.47 ±6.61	31.58 ±5.07	33.57 ±6.43	29.94 ±4.84	33.34 ±7.13
tangential length width (μm)	25.81 ±5.54	23.59 ±4.48	24.24 ±4.82	22.76 ±4.57	26.21 ±6.7
percent cell wall area (%(μm^2)	39.64	49.36	54.55	43.39	35.94
Existing cells					
lumen area (μm2)	631.45 ±251.75	495.85 ±231.74	748.59 ±290.11	623.96 ±268.93	780.79 ±365.27
radial length (μm)	32.54 ±5.91	30.86 ±6.016	35.82 ±6.37	35.25 ±7.2	36.48 ±8.08
tangential length width (μm)	24.06 ±6.17	20.25 ±6.69	25.92 ±6.19	22.24 ±5.97	25.52 ±7.62
percent cell wall area (%(μm^2)	32.76	34.05	35.93	35.48	31.83
percent lignin area in CML/S1	8.66	19.54	22.96	21.02	19.21

Set number 3 culture date: 31st March 04 Growth period: 1 month Rotorua	0.003 mM NAA	0.03 mM NAA	0.3 mM NAA	3 mM NAA	Control
Changes in cell number					
Cambial region cell number	4.4 ±0.89	6 ±1.09	5.2 ±0.84	5.17 ±1.33	4 ±0.82
RE region cell number	20 ±2	16.83333 ±4.792355	23.25 ±2.774887	19 ±3.162278	22.75 ±1.892969
Developing cells region	19.33 ±1.37	17.33 ±1.21	23.8 ±1.92	19.5 ±2.07	14.25 ±0.96
Cambial region cells					
lumen area (μm2)	215.61 ±87.38	162.09 ±52.19	200.31 ±73.99	200.31 ±73.99	190.40 ±52.78
radial length (μm)	7.43 ±1.75	6.13 ±1.47	6.61 ±1.48	6.61 ±1.48	6.76 ±1.48
tangential length width (μm)	28.28 ±6.01	26.34 5.99	29.80 ±6.72	29.80 ±6.72	28.148 ±5.26
RE region cells					
lumen area (μm2)	810.19 ±256.71	554.62 ±218.47	562.84 ±218.29	493.26 ±186.98	672.51 ±250.01
radial length (μm)	36.51 ±5.88	30.22 ±5.87	29.57 ±6.14	27.64 ±6.09	33.18 ±6.38
tangential length width (μm)	28.48 ±5.9	23.11 ±5.17	23.63 ±4.64	22.02 ±4.96	25.89 ±5.85
percent cell wall area (%(μm^2)	39.46	41.21	61.11	74.59	66.38
Existing cells					
lumen area (μm2)	695.88 ±268.05	721.94 ±276.96	777.69 ±344.59	799.2 ±370.61	534.76 ±239.01
radial length (μm)	35.33 ±6.48	34.76 ±6.73	40.08 ±6.88	38.1 ±7.48	31 ±5.73
tangential length width (μm)	24.54 ±6.22	25.8 ±6.42	23.71 ±6.89	25.75 ±7.68	21.03 ±6.49
percent cell wall area (%(μm^2)	31.06	28.39	22.73	26.05	32.27
percent lignin area in CML/S1	13.35		18.91		14.17

Statistical analysis for data presented in chapter six

For ease of the analysis the treatments were coded with numbers from 1 to 5

Code 1- 0.003 mM auxin concentration
Code 2- 0.03 mM auxin concentration } Low auxin levels
Code 3- 0.3 mM auxin concentration
Code 4- 3 mM auxin concentration } high auxin levels
Code 5- control.

Auxin treated cultured wood cambial cell count analysis

Completely Randomized AOV for cambial

Source	DF	SS	MS	F	P
sample	4	37.091	9.27283	6.32	0.0002
Error	79	115.861	1.46660		
Total	83	152.952			

Grand Mean 4.4762 CV 27.05

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	14.6	4	0.0057
Cochran's Q	0.4765		
Largest Var / Smallest Var	4.7777		

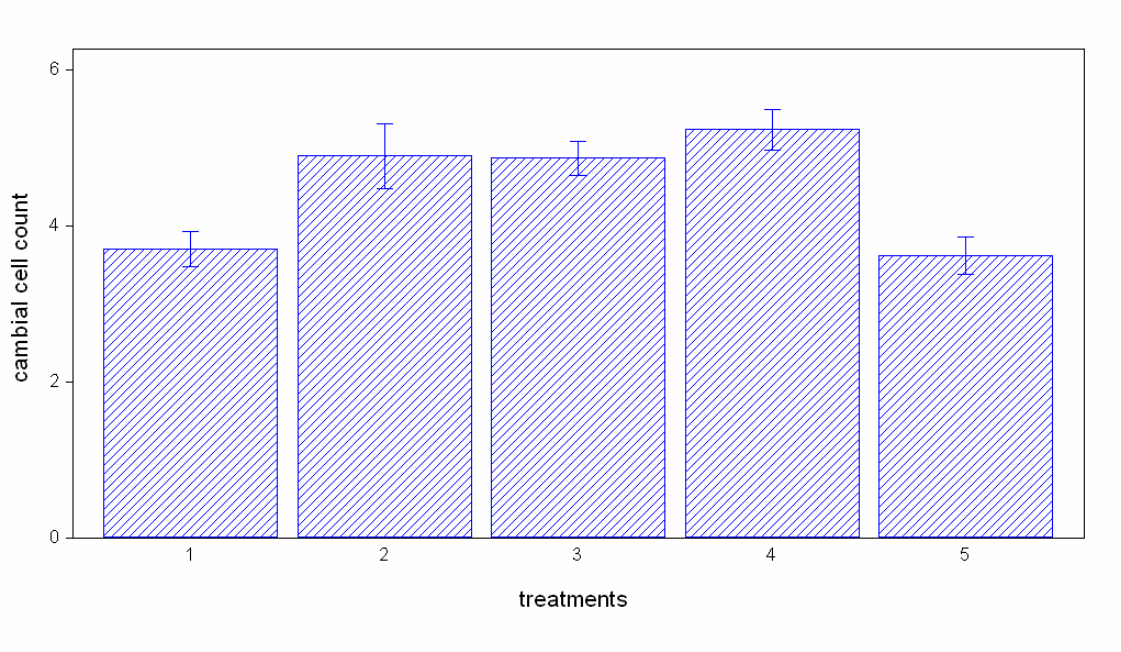
Component of variance for between groups 0.46538
Effective cell size 16.8

sample	N	Mean	SE
1	17	3.7059	0.2937
2	19	4.8947	0.2778
3	15	4.8667	0.3127
4	17	5.2353	0.2937
5	16	3.6250	0.3028

Tukey HSD All-Pairwise Comparisons Test of cambial by sample

sample	Mean	Homogeneous Groups
4	5.2353	A
2	4.8947	A
3	4.8667	AB
1	3.7059	BC
5	3.6250	C

Alpha 0.05
Critical Q Value 3.948
There are 3 groups (A, B, etc.) in which the means
are not significantly different from one another.



Auxin treated cultured wood RE cell count anaylsis

Completely Randomized AOV for RE

Source	DF	SS	MS	F	P
sample	4	205.62	51.4048	3.67	0.0086
Error	79	1107.94	14.0246		
Total	83	1313.56			

Grand Mean 20.869 CV 17.94

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	23.3	4	0.0001
Cochran's Q	0.3572		
Largest Var / Smallest Var	10.929		

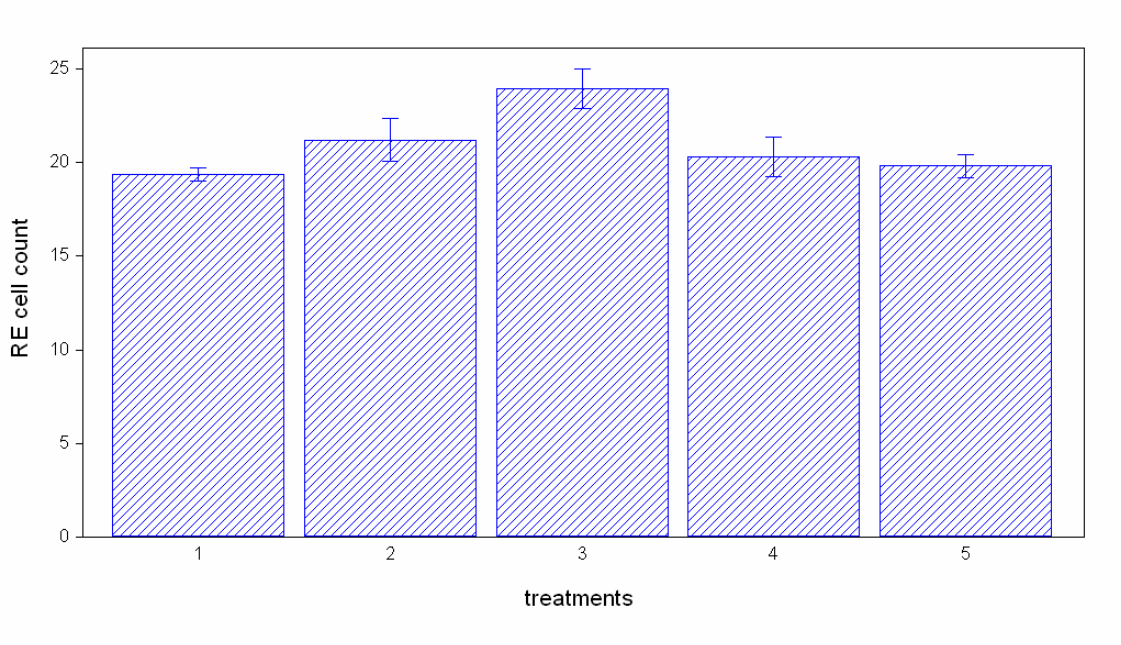
Component of variance for between groups 2.22849
Effective cell size 16.8

sample	N	Mean	SE
1	17	19.353	0.9083
2	19	21.211	0.8591
3	15	23.933	0.9669
4	17	20.294	0.9083
5	16	19.813	0.9362

Tukey HSD All-Pairwise Comparisons Test of RE by sample

sample	Mean	Homogeneous Groups
3	23.933	A
2	21.211	AB
4	20.294	AB
5	19.813	B
1	19.353	B

Alpha 0.05
Critical Q Value 3.948
There are 2 groups (A and B) in which the means
are not significantly different from one another.



Auxin treated cultured wood developing cell count analysis

Completely Randomized AOV for N

Source	DF	SS	MS	F	P
sample	4	217.60	54.3993	5.01	0.0012
Error	79	857.17	10.8503		
Total	83	1074.77			

Grand Mean 16.864 CV 19.53

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	38.1	4	0.0000

Cochran's Q 0.4816
Largest Var / Smallest Var 25.259

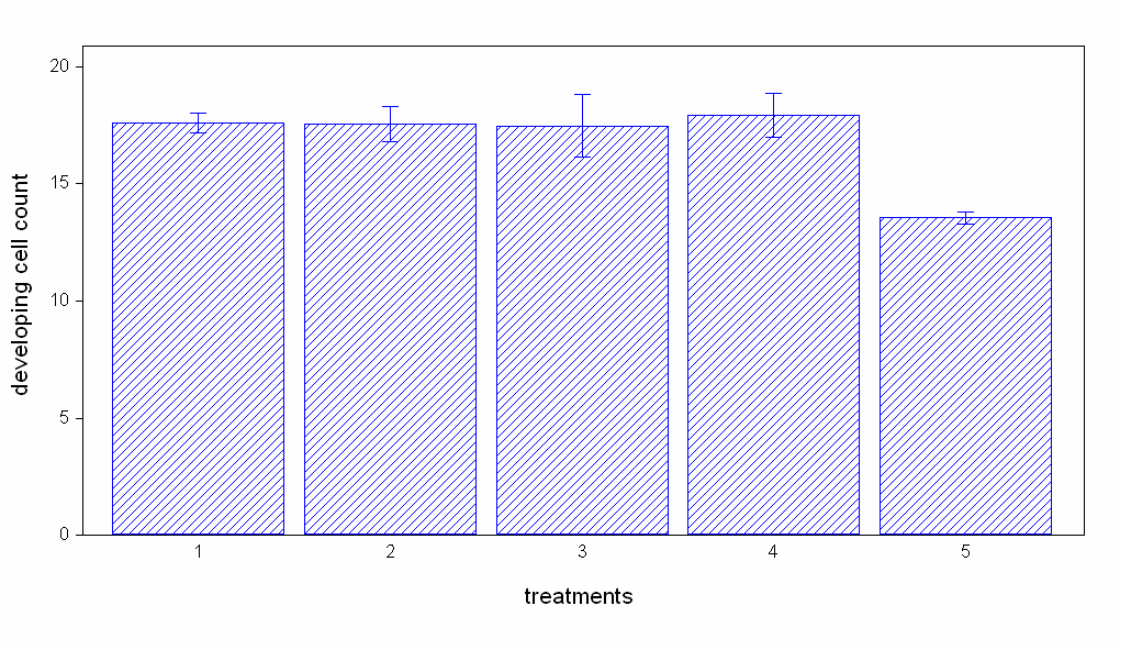
Component of variance for between groups 2.59625
Effective cell size 16.8

sample	N	Mean	SE
1	17	17.588	0.7989
2	19	17.556	0.7557
3	15	17.467	0.8505
4	17	17.941	0.7989
5	16	13.562	0.8235

Tukey HSD All-Pairwise Comparisons Test of N by sample

sample	Mean	Homogeneous Groups
4	17.941	A
1	17.588	A
2	17.556	A
3	17.467	A
5	13.562	B

Alpha 0.05
Critical Q Value 3.948
There are 2 groups (A and B) in which the means
are not significantly different from one another.



Auxin treated cultured wood cambial cell lumen area analysis

Completely Randomized AOV for area

Source	**DF**	**SS**	**MS**	**F**	**P**
sample	4	91474	22868.4	5.54	0.0003
Error	295	1217565	4127.3		
Total	299	1309039			

Grand Mean 176.85 CV 36.33

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	12.3	4	0.0149
Cochran's Q	0.2806		
Largest Var / Smallest Var	2.1420		

Component of variance for between groups 312.352
Effective cell size 60.0

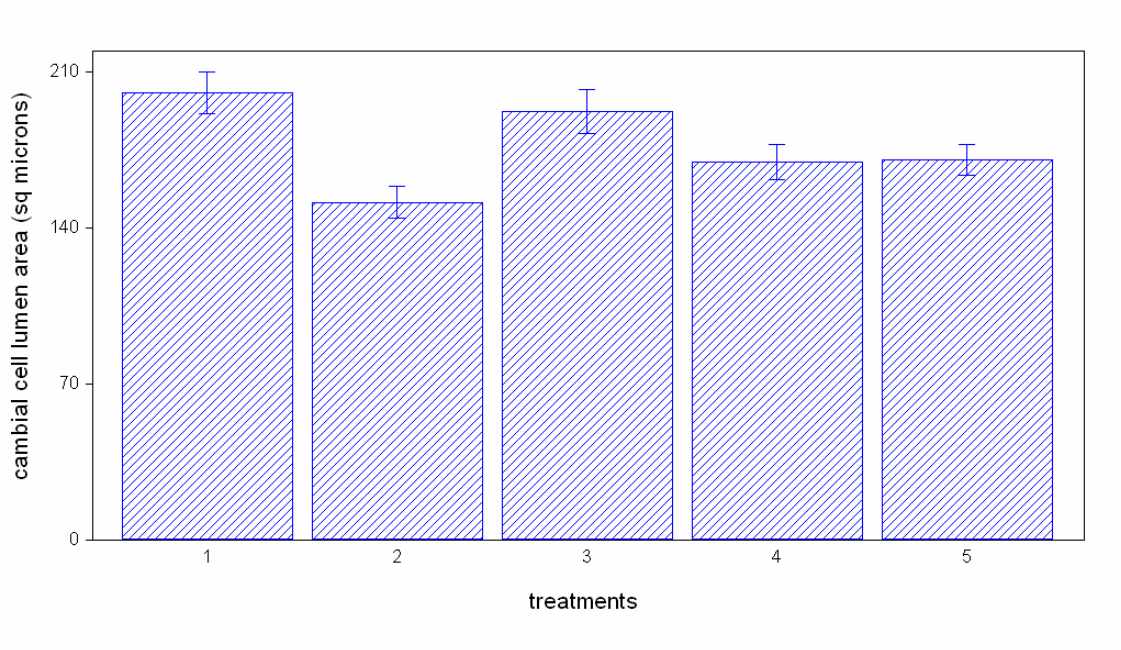
sample	**Mean**
1	200.47
2	151.53
3	192.12
4	169.60
5	170.52

Observations per Mean 60
Standard Error of a Mean 8.2939
Std Error (Diff of 2 Means) 11.729

Tukey HSD All-Pairwise Comparisons Test of area by sample

sample	Mean	Homogeneous Groups
1	200.47	A
3	192.12	A
5	170.52	AB
4	169.60	AB
2	151.53	B

Alpha 0.05 Standard Error for Comparison 11.729
Critical Q Value 3.857 Critical Value for Comparison 31.987
There are 2 groups (A and B) in which the means
are not significantly different from one another.



Auxin treated cultured wood cambial cell radial length analysis

Completely Randomized AOV for radial

Source	DF	SS	MS	F	P
sample	4	58.620	14.6549	5.90	0.0001
Error	295	732.544	2.4832		
Total	299	791.163			

Grand Mean 6.4856 CV 24.30

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	5.53	4	0.2370

Cochran's Q 0.2337
Largest Var / Smallest Var 1.5677

Component of variance for between groups 0.20286
Effective cell size 60.0

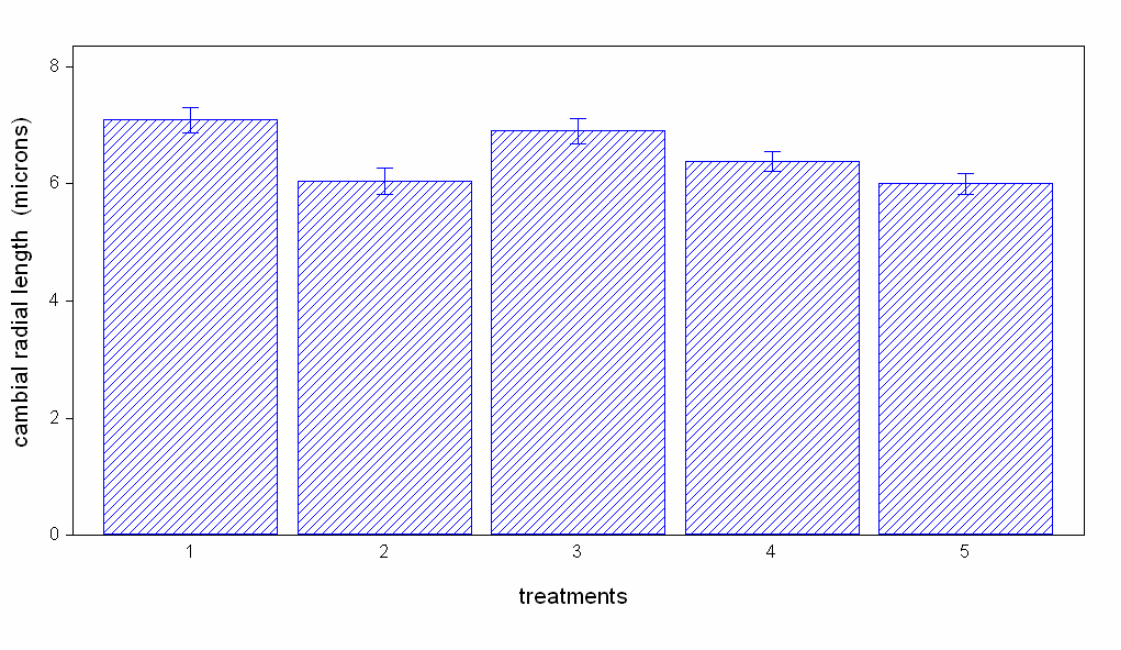
sample	Mean
1	7.0892
2	6.0449
3	6.9048
4	6.3861
5	6.0031

Observations per Mean 60
Standard Error of a Mean 0.2034
Std Error (Diff of 2 Means) 0.2877

Tukey HSD All-Pairwise Comparisons Test of radial by sample

sample	Mean	Homogeneous Groups
1	7.0892	A
3	6.9048	A
4	6.3861	AB
2	6.0449	B
5	6.0031	B

Alpha 0.05 Standard Error for Comparison 0.2877
Critical Q Value 3.857 Critical Value for Comparison 0.7846
There are 2 groups (A and B) in which the means
are not significantly different from one another.



Auxin treated cambial tangential length analysis

Completely Randomized AOV for tangential

Source	DF	SS	MS	F	P
sample	4	507.2	126.805	3.44	0.0091
Error	295	10885.1	36.899		
Total	299	11392.3			

Grand Mean 26.965 CV 22.53

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	6.71	4	0.1523
Cochran's Q	0.2657		
Largest Var / Smallest Var	1.8258		

Component of variance for between groups 1.49844
Effective cell size 60.0

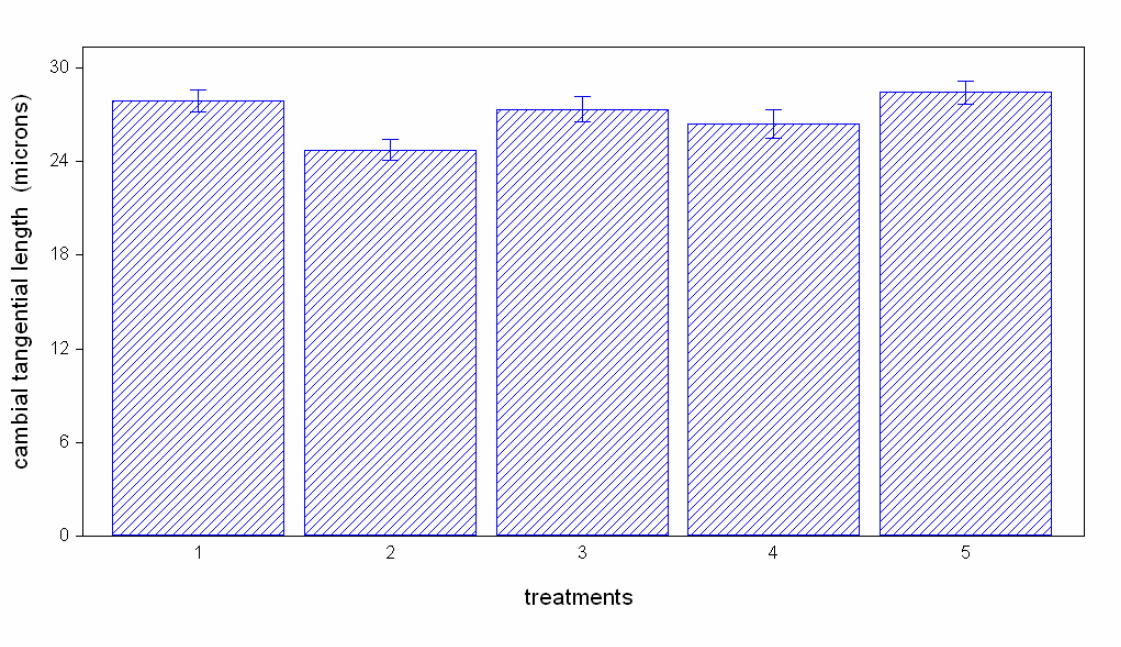
sample	Mean
1	27.893
2	24.724
3	27.359
4	26.427
5	28.423

Observations per Mean 60
Standard Error of a Mean 0.7842
Std Error (Diff of 2 Means) 1.1090

Tukey HSD All-Pairwise Comparisons Test of tangential by sample

sample	Mean	Homogeneous Groups
5	28.423	A
1	27.893	A
3	27.359	AB
4	26.427	AB
2	24.724	B

Alpha 0.05 Standard Error for Comparison 1.1090
Critical Q Value 3.857 Critical Value for Comparison 3.0244
There are 2 groups (A and B) in which the means
are not significantly different from one another.



Auxin treated cultured wood developing cells, cell lumen area analysis

Completely Randomized AOV for cell

Source	DF	SS	MS	F	P
samples	4	5074762	1268691	24.1	0.0000
Error	1298	6.840E+07	52693		
Total	1302	7.347E+07			

Grand Mean 613.92 CV 37.39

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	24.7	4	0.0001

Cochran's Q 0.2389
Largest Var / Smallest Var 1.5486

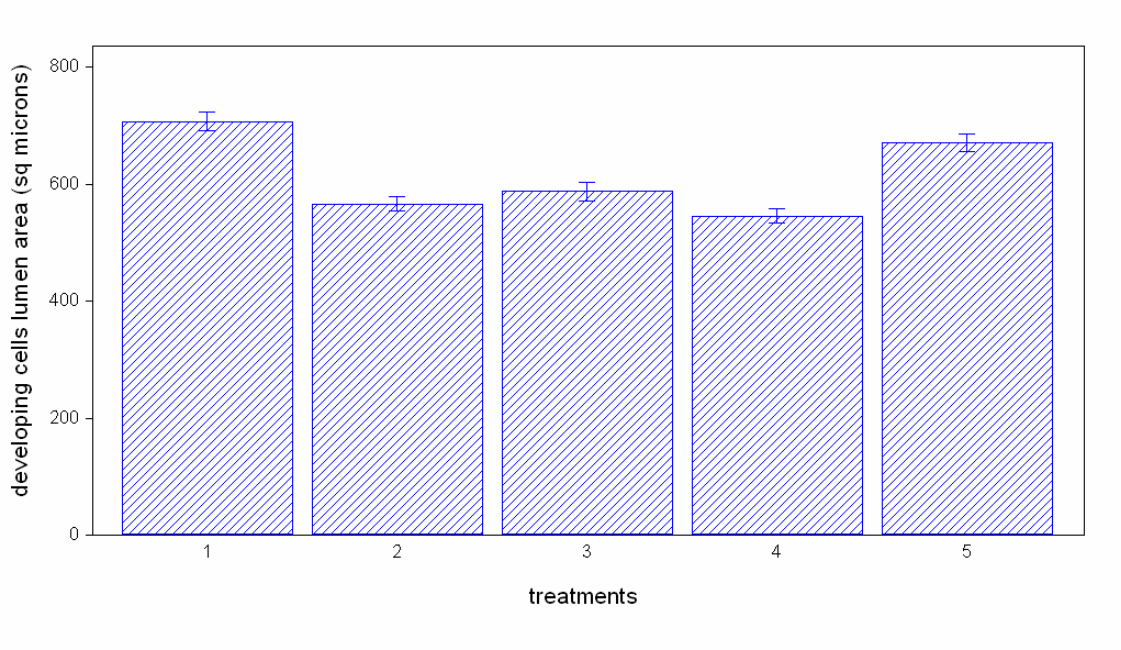
Component of variance for between groups 4668.77
Effective cell size 260.5

samples	N	Mean	SE
1	246	706.70	14.636
2	276	565.94	13.817
3	246	587.17	14.636
4	266	544.88	14.075
5	269	671.04	13.996

Tukey HSD All-Pairwise Comparisons Test of cell by samples

samples	Mean	Homogeneous Groups
1	706.70	A
5	671.04	A
3	587.17	B
2	565.94	B
4	544.88	B

Alpha 0.05
Critical Q Value 3.857
There are 2 groups (A and B) in which the means
are not significantly different from one another.



Auxin treated cultured wood developing cells radial length analysis

Completely Randomized AOV for radial

Source	**DF**	**SS**	**MS**	**F**	**P**
samples	4	2818.8	704.703	17.0	0.0000
Error	1298	53836.7	41.477		
Total	1302	56655.6			

Grand Mean 32.226 CV 19.98

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	53.5	4	0.0000
Cochran's Q	0.3227		
Largest Var / Smallest Var	2.3012		

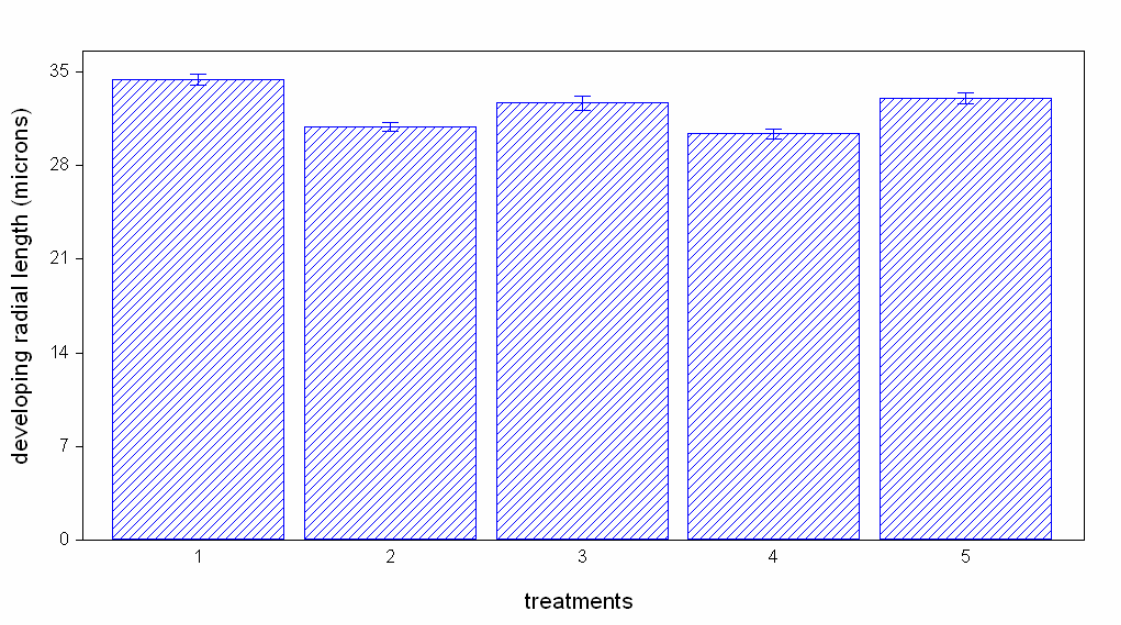
Component of variance for between groups 2.54643
Effective cell size 260.5

samples	**N**	**Mean**	**SE**
1	246	34.411	0.4106
2	276	30.879	0.3877
3	246	32.690	0.4106
4	266	30.367	0.3949
5	269	33.023	0.3927

Tukey HSD All-Pairwise Comparisons Test of radial by samples

samples	Mean	Homogeneous Groups
1	34.411	A
5	33.023	AB
3	32.690	B
2	30.879	C
4	30.367	C

Alpha 0.05
Critical Q Value 3.857
There are 3 groups (A, B, etc.) in which the means
are not significantly different from one another.



Auxin treated cultured wood developing cells tangential length analysis

Completely Randomized AOV for tangetial

Source	DF	SS	MS	F	P
samples	4	2626.5	656.627	22.7	0.0000
Error	1298	37555.3	28.933		
Total	1302	40181.8			

Grand Mean 24.491 CV 21.96

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	14.6	4	0.0056
Cochran's Q	0.2412		
Largest Var / Smallest Var	1.5387		

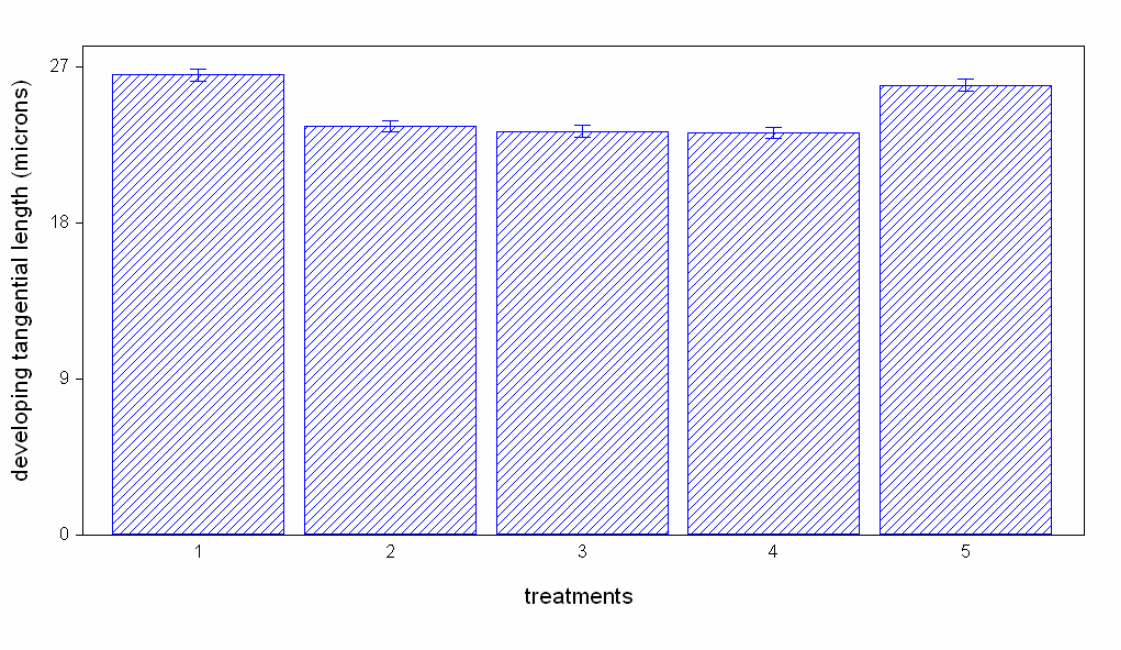
Component of variance for between groups 2.41000
Effective cell size 260.5

samples	N	Mean	SE
1	246	26.540	0.3429
2	276	23.578	0.3238
3	246	23.272	0.3429
4	266	23.211	0.3298
5	269	25.937	0.3280

Tukey HSD All-Pairwise Comparisons Test of tangetial by samples

samples	Mean	Homogeneous Groups
1	26.540	A
5	25.937	A
2	23.578	B
3	23.272	B
4	23.211	B

Alpha 0.05
Critical Q Value 3.857
There are 2 groups (A and B) in which the means
are not significantly different from one another.



Auxin treated cultured wood existing cell, cell lumen area analysis

Completely Randomized AOV for Area

Source	DF	SS	MS	F	P
sample	4	3228975	807244	7.66	0.0000
Error	1371	1.445E+08	105398		
Total	1375	1.477E+08			

Grand Mean 716.82 CV 45.29

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	26.4	4	0.0000
Cochran's Q	0.2477		
Largest Var / Smallest Var	1.7939		

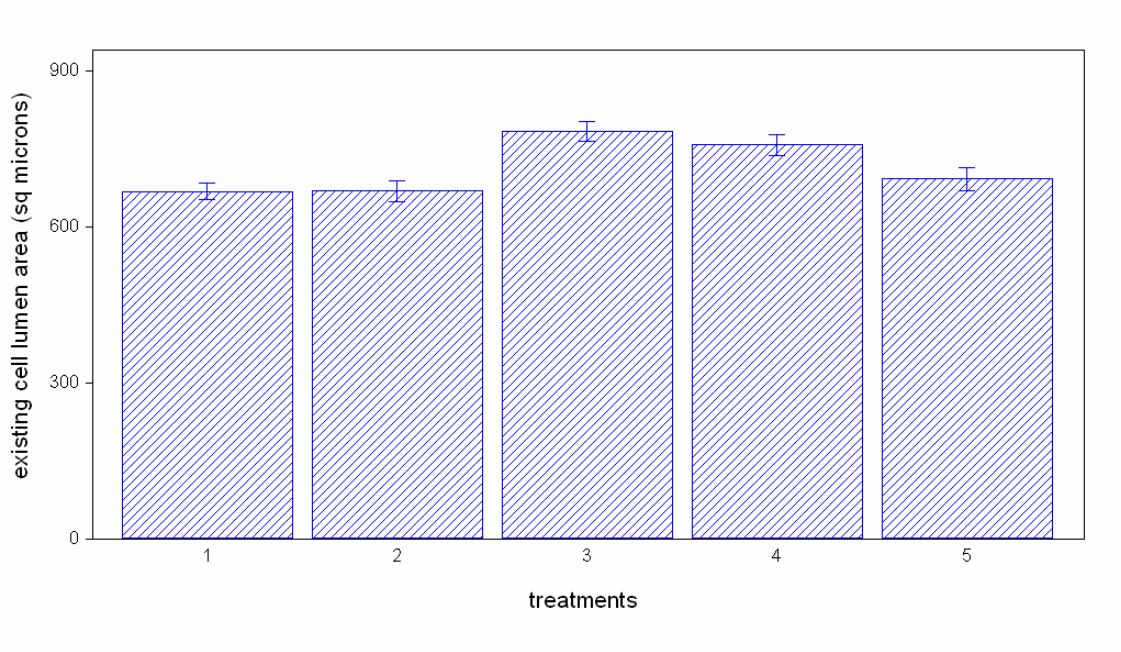
Component of variance for between groups 2554.74
Effective cell size 274.7

sample	N	Mean	SE
1	286	667.94	19.197
2	237	668.79	21.088
3	304	783.84	18.620
4	285	757.41	19.231
5	264	691.91	19.981

Tukey HSD All-Pairwise Comparisons Test of Area by sample

sample	Mean	Homogeneous Groups
3	783.84	A
4	757.41	AB
5	691.91	BC
2	668.79	C
1	667.94	C

Alpha 0.05
Critical Q Value 3.857
There are 3 groups (A, B, etc.) in which the means
are not significantly different from one another.



Auxin treated cultured wood existing cell radial length analysis

Completely Randomized AOV for radial

Source	DF	SS	MS	F	P
sample	4	3197.5	799.377	14.0	0.0000
Error	1371	78425.1	57.203		
Total	1375	81622.6			

Grand Mean 35.900 CV 21.07

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	9.71	4	0.0455

Cochran's Q 0.2471
Largest Var / Smallest Var 1.3664

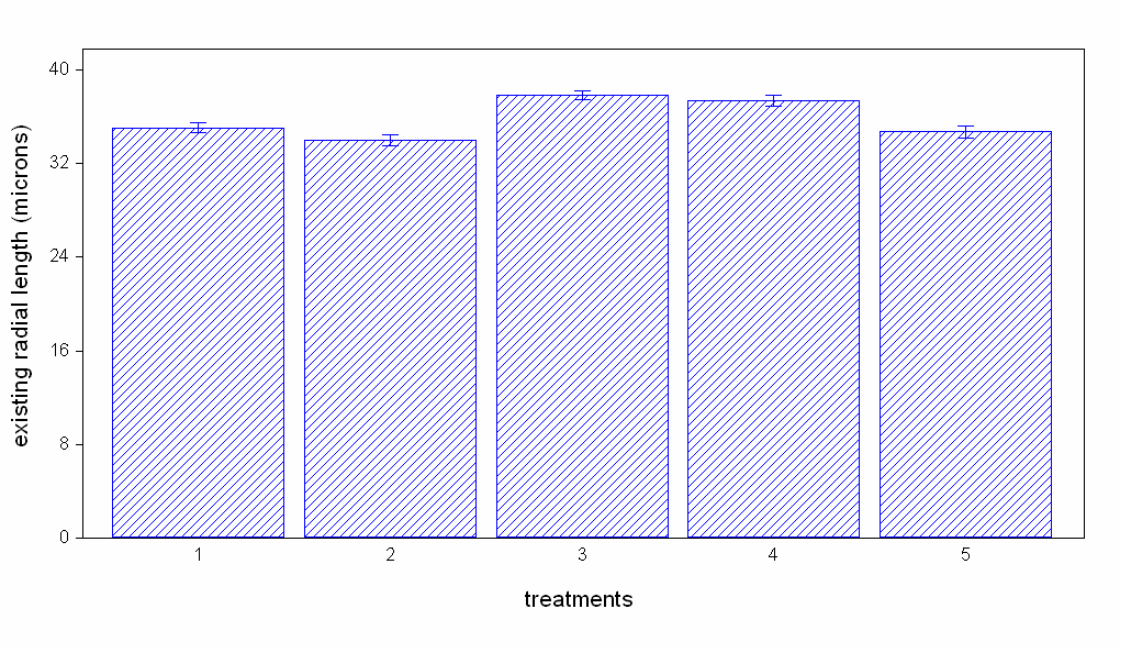
Component of variance for between groups 2.70154
Effective cell size 274.7

sample	N	Mean	SE
1	286	35.063	0.4472
2	237	33.963	0.4913
3	304	37.841	0.4338
4	285	37.357	0.4480
5	264	34.736	0.4655

Tukey HSD All-Pairwise Comparisons Test of radial by sample

sample	Mean	Homogeneous Groups
3	37.841	A
4	37.357	A
1	35.063	B
5	34.736	B
2	33.963	B

Alpha 0.05
Critical Q Value 3.857
There are 2 groups (A and B) in which the means
are not significantly different from one another.



Auxin treated cultured wood existing cell tangential length analysis

Completely Randomized AOV for tangentia

Source	DF	SS	MS	F	P
sample	4	692.1	173.015	3.36	0.0095
Error	1371	70589.1	51.487		
Total	1375	71281.2			

Grand Mean 24.625 CV 29.14

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	16.7	4	0.0022

Cochran's Q 0.2258
Largest Var / Smallest Var 1.4439

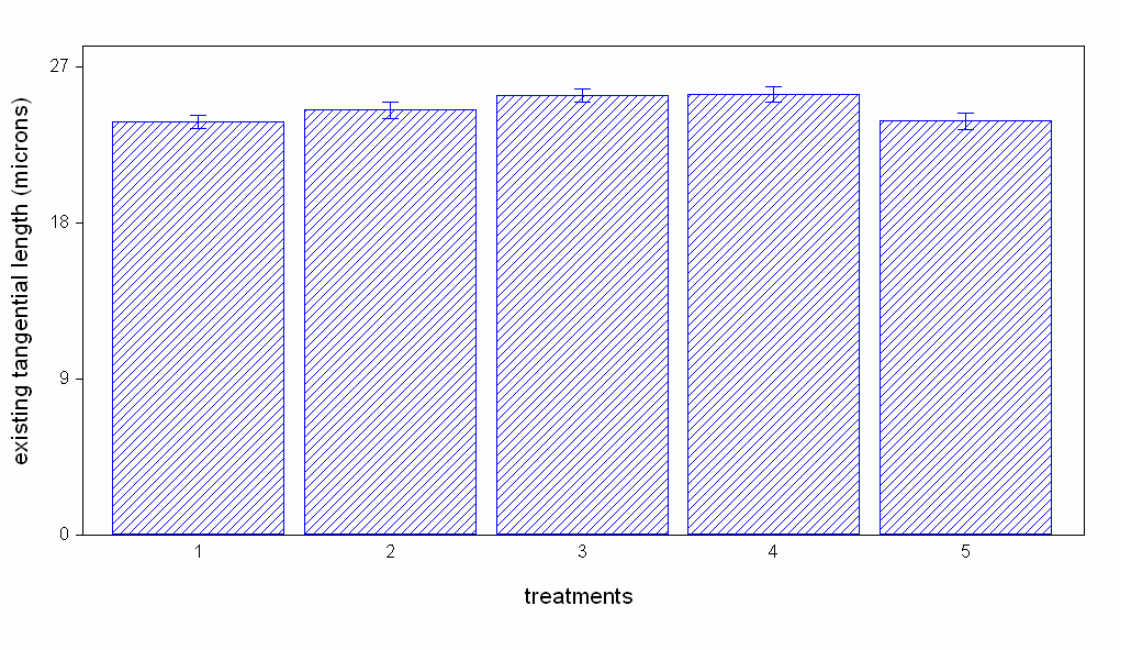
Component of variance for between groups 0.44236
Effective cell size 274.7

sample	N	Mean	SE
1	286	23.820	0.4243
2	237	24.503	0.4661
3	304	25.378	0.4115
4	285	25.425	0.4250
5	264	23.875	0.4416

Tukey HSD All-Pairwise Comparisons Test of tangentia by sample

sample	Mean	Homogeneous Groups
4	25.425	A
3	25.378	A
2	24.503	A
5	23.875	A
1	23.820	A

Alpha 0.05
Critical Q Value 3.857
There are no significant pairwise differences among the means.



Auxin treated cultured cell %cell wall thickness of developing cells

Completely Randomized AOV for C

Source	**DF**	**SS**	**MS**	**F**	**P**
sample	4	1214.45	303.613	1.36	0.3154
Error	10	2236.03	223.603		
Total	14	3450.49			

Grand Mean 44.587 CV 33.54

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	0.59	4	0.9646
Cochran's Q	0.2868		
Largest Var / Smallest Var	3.2381		

Component of variance for between groups 26.6700
Effective cell size 3.0

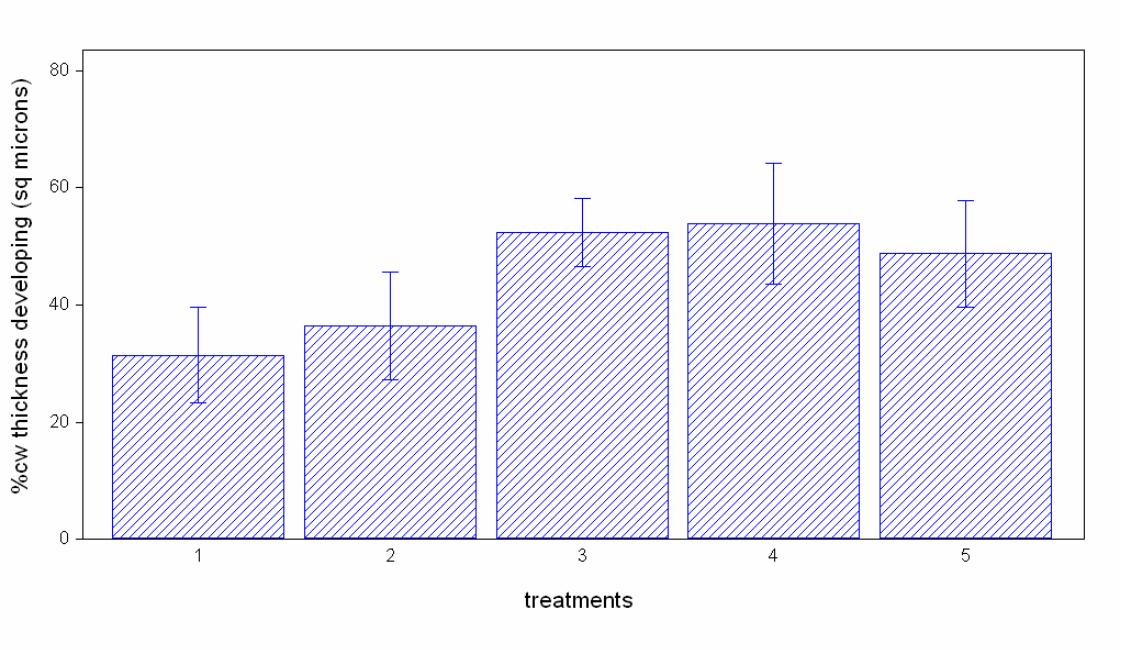
sample	**Mean**
1	31.438
2	36.431
3	52.403
4	53.913
5	48.751

Observations per Mean 3
Standard Error of a Mean 8.6333
Std Error (Diff of 2 Means) 12.209

Tukey HSD All-Pairwise Comparisons Test of C by sample

sample	Mean	Homogeneous Groups
4	53.913	A
3	52.403	A
5	48.751	A
2	36.431	A
1	31.438	A

Alpha 0.05 Standard Error for Comparison 12.209
Critical Q Value 4.655 Critical Value for Comparison 40.185
There are no significant pairwise differences among the means.



Auxin treated cultured wood existing cells %cell wall thickness analysis

Completely Randomized AOV for C

Source	DF	SS	MS	F	P
samples	4	95.31	23.828	0.20	0.9307
Error	10	1171.31	117.131		
Total	14	1266.62			

Grand Mean 36.322 CV 29.80

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	3.42	4	0.4898
Cochran's Q	0.4158		
Largest Var / Smallest Var	19.424		

Component of variance for between groups -31.1012
Effective cell size 3.0

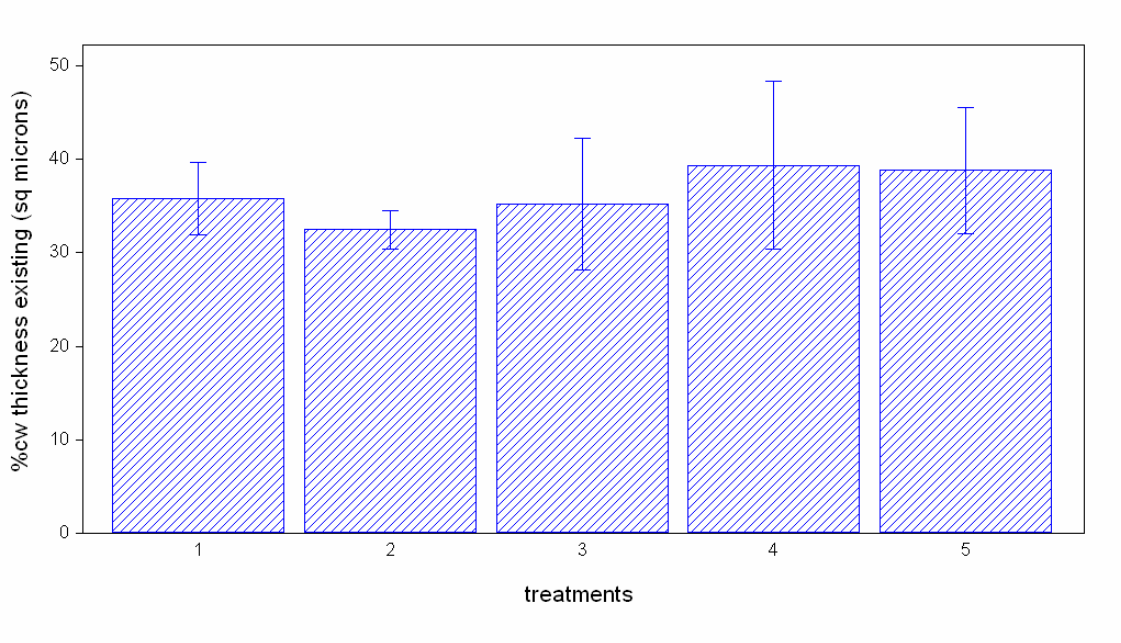
samples	Mean
1	35.803
2	32.457
3	35.203
4	39.357
5	38.792

Observations per Mean 3
Standard Error of a Mean 6.2485
Std Error (Diff of 2 Means) 8.8367

Tukey HSD All-Pairwise Comparisons Test of C by samples

samples	Mean	Homogeneous Groups
4	39.357	A
5	38.792	A
1	35.803	A
3	35.203	A
2	32.457	A

Alpha 0.05 Standard Error for Comparison 8.8367
Critical Q Value 4.655 Critical Value for Comparison 29.085
There are no significant pairwise differences among the means.



Auxin treated cultured wood %lignin area observed with epifluorescence microscopy

Completely Randomized AOV for C

Source	DF	SS	MS	F	P
samples	4	1688.76	422.190	10.6	0.0000
Error	59	2349.50	39.822		
Total	63	4038.26			

Grand Mean 18.549 CV 34.02

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	10.0	4	0.0403

Cochran's Q 0.3353
Largest Var / Smallest Var 4.6501

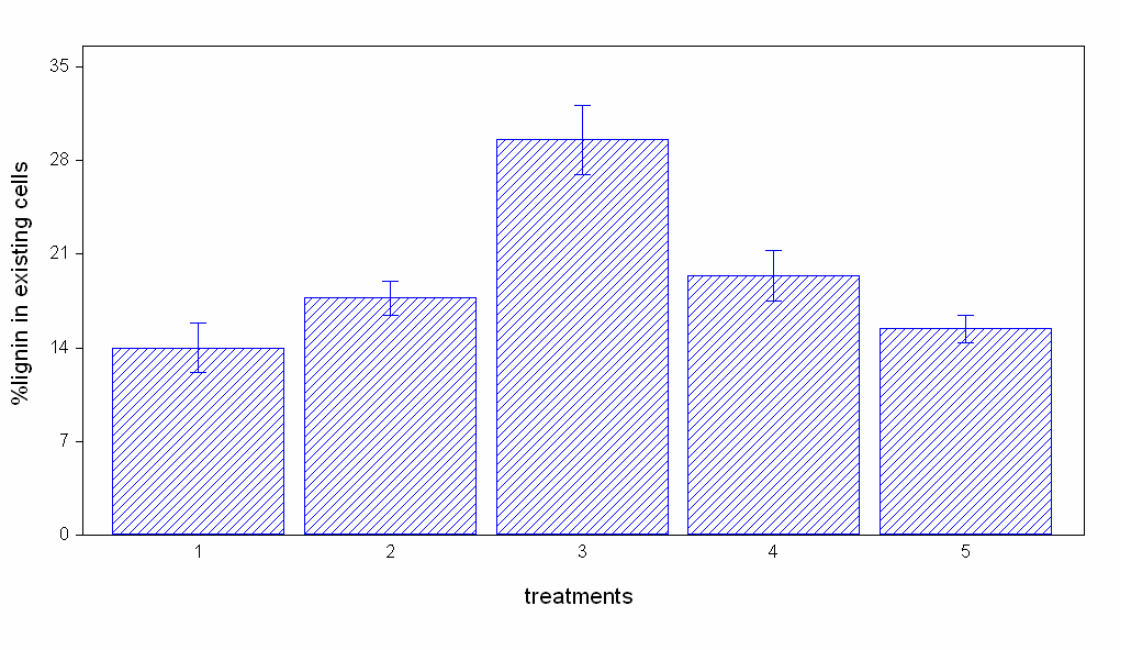
Component of variance for between groups 30.2118
Effective cell size 12.7

samples	N	Mean	SE
1	15	13.993	1.6294
2	9	17.722	2.1035
3	10	29.561	1.9955
4	15	19.396	1.6294
5	15	15.411	1.6294

Tukey HSD All-Pairwise Comparisons Test of C by samples

samples	Mean	Homogeneous Groups
3	29.561	A
4	19.396	B
2	17.722	B
5	15.411	B
1	13.993	B

Alpha 0.05
Critical Q Value 3.980
There are 2 groups (A and B) in which the means
are not significantly different from one another.



Auxin treated cultured wood acetyl bromide lignin assay analysis

Completely Randomized AOV for lignin

Source	**DF**	**SS**	**MS**	**F**	**P**
samples	4	15.044	3.7610	0.28	0.8828
Error	9	120.325	13.3694		
Total	13	135.369			

Grand Mean 21.513 CV 17.00

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	2.00	4	0.7365
Cochran's Q	0.5393		
Largest Var / Smallest Var	6.8666		

Component of variance for between groups -3.44917
Effective cell size 2.8

samples	**N**	**Mean**	**SE**
1	3	20.050	2.1110
2	3	21.188	2.1110
3	3	22.912	2.1110
4	3	21.210	2.1110
5	2	22.552	2.5855

Tukey HSD All-Pairwise Comparisons Test of lignin by samples

samples	Mean	Homogeneous Groups
3	22.912	A
5	22.552	A
4	21.210	A
2	21.188	A
1	20.050	A

Alpha 0.05
Critical Q Value 4.752
There are no significant pairwise differences among the means.

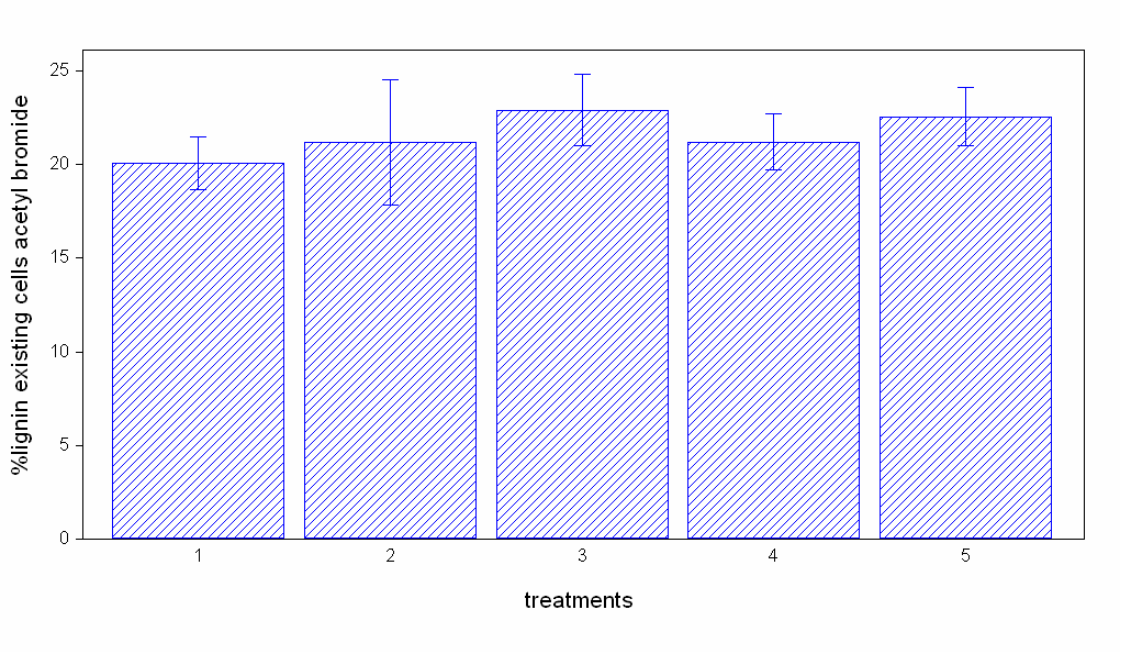


Table 5 *: Composition of lignin and polysaccharides breakdown products identified by the Py-GC-MS. The results are the averages of the duplicate measurements performed on one set of cultures. Values are ± standard error of mean.*

	Auxin treatments		
Carbohydrate Products	0.003 mM NAA	3 mM NAA	Control
4-hydroxy-5,6-dihydro-(2H)-pyran-2-one	16.483 ±0.46	2.441 ±0.26	7 ±0.02
HMF	34.229 ±0.56	6.836 ±0.07	2 ±0.03
4-allyl phenol	1.127 ±0.14	0.249 ±0.02	0.02 ±0.04
anhydro gluco pyranose	29.235 ±6.65	11.605 ±0.9	9 ±0.5

	Auxin treatments		
Lignin Products	0.003 mM NAA	3 mM NAA	Control
guaiacol	29.379 ±3.73	22.326 ±0.51	19 ±0.01
2-methyl phenol	2.802 ±0.31	2.181 ±0.22	2 ±0.4
phenol	10.006 ±0.5	8.913 ±0.44	7 ±1
4-vinyl guaiacol	20.618 ±0.75	20.295 ±0.43	18 ±0.1
dihydro coniferyl alcohol	2.257 ±0.5	3.222 ±0.08	2 ±0.1
coniferaldehyde	8.878 ±1.82	10.934 ±0.33	6 ±0.6
coniferyl alcohol	4.005 ±1.22	4.517 ±0.44	2 ±0.4
vanillin	10.544 ±0.04	11.74 ±0.2	8 ±0.5
eugenol	11.511 ±1.7	15.872 ±0.3	10 ±0.1

Appendix Five

Supplemental auxin and boron culture analysis

The appendix presents supplemental culture analysis and statistical analysis for the data presented in chapter seven.

Table 4: The details of the measurements of the three sets of repeats that were cultured for each treatment from six different trees and treated with different concentrations of auxin and boron.

Set No. 1 culture date: 21ST Jan 05 Burnham tree Growth Period: 2 months	1 µM B, 0.03 mM NAA	7 µM B, 0.03 mM NAA	25 µM B, 0.03 mM NAA	100 µM B, 0.03 mM NAA	control
Changes in cell number					
Cambial region	3.67 ±0.82	3.83 ±0.75	4.4 ±0.89	5 ±1.58	2.29 ±0.49
RE region	17.83 ±1.169	16.833 ±3.061	15.4 ±1.817	12.4 ±2.51	12.714 ±1.113
Developing cells region	15.833 ±2.858	20.833 ±1.602	12.8 ±1.789	16.6 ±1.517	15.167 ±2.229
Cambial region cells					
lumen area (μm^2)	305.287 ±135.881	297.946 ±89.746	273.168 ±107.979	249.676 ±97.98	236.014 ±75.139
radial length (µm)	9.657 ±2.779	9.939 ±2.235	9.441 ±2.727	7.86 ±2.082	8.096 ±1.715
tangential length (µm)	30.765 ±7.791	29.713 ±5.436	28.674 ±6.166	31.122 ±5.943	28.663 ±5.269
RE region cells					
lumen area (μm^2)	/	1092.362 ±424.7579	935.7273 ±464.3472	861.8972 ±313.1842	876.5759 ±371.4378
radial length (µm)	/	41.33447 ±7.771748	38.99556 ±9.967131	38.58268 ±7.306977	38.24699 ±7.225497
tangential length (µm)	/	32.85243 ±7.091835	29.13728 ±8.308957	29.24321 ±6.519584	28.62739 ±7.757109
cell wall area(%μm^2)	34.985	20.304	43.26	24.868	34.891

Existing cells					
lumen area (μm^2)	1066.005 ±462.092	817.789 ±348.56	859.063 ±337.729	839.331 ±391.325	794.253 ±343.037
radial length (μm)	42.0785 ±8.636	36.353 ±7.228	41.512 ±8.872	40.962 ±9.256	37.864 ±7.836
tangential length (μm)	31.31 ±8.393	27.748 ±7.234	26.323 ±6.6	26.476 ±6.907	25.991 ±6.749
cell wall area(%μm^2)	24.71009	27.70931	17.51043	8.574766	22.87914
lignin area in CML/S_1(% lignin μm^2)	5.432599	/	/	5.573531	4.384288
Set No. 2 culture date 26th Apr 04 Rotorua tree Growth Period: 1 month	1 μM B, 0.03 mM NAA	7 μM B, 0.03 mM NAA	25 μM B, 0.03 mM NAA	100 μM B, 0.03 mM NAA	control
Changes in cell number					
Cambial region	3 ±0.63	3.2 ±0.45	3.8 ±0.84	4.4 ±1.14	2 ±0
RE region	23.167 ±2.64	21.6 ±3.44	27.6 ±4.723	27.6 ±3.65	25.167 ±2.563
Developing cells region	12.833 ±1.834848	17.25 ±1.517	17.4 ±2.074	16.2 ±0.837	14 ±1.414
Cambial region cells					
lumen area (μm^2)	208.38 ±87.21	217.6 ±85.1	171.91 ±67.09	184.61 ±94.25	208.35 ±68.93
radial length (μm)	8.34 ±2.35	9.45 ±0.97	7.57 ±1.85	7.07 ±2.22	7.16 ±2.14
tangential length (μm)	26.7 ±6.01	27.02 ±5.07	22.15 ±4.51	25.04 ±7.09	29.18 ±4.85
RE region cells					
lumen area (μm^2)	605.167 ±203.4102	625.1523 ±238.6811	683.1657 ±249.8917	734.5431 ±267.9229	±
radial length (μm)	31.56366 ±5.724631	33.04704 ±5.705219	33.13981 ±6.20473	35.47336 ±6.420677	±
tangential length (μm)	24.54156 ±5.419131	24.19333 ±6.640416	25.8172 ±5.752273	26.56477 ±6.179439	±
cell wall area(%μm^2)	23.972	24.268	28.26	29.387	16.69
Existing cells					
lumen area (μm^2)	676 ±360.077	611.578 ±266.209	583.102 ±230.777	704.942 ±279.49	678.536 ±264.266

radial length (μm)	35.048 ±11.198	32.087 ±6.313	32.346 ±5.292	35.76 ±6.957	36 ±8.64
tangential length (μm)	23.32 ±7.207	23.571 ±6.004	22.471 ±5.993	24.144 ±6.28	23.83 ±6.78
cell wall area(%μm^2)	20.55367	21.9079	20.60217	24.12292	14.34707
lignin area in CML/S_1(% lignin μm^2)	4.659833	3.517914	3.305273	3.944884	3.622167
Set No. 3 culture date 25th March 05 Growth Period: 1 month	1 μM B, 0.03 mM NAA	7 μM B, 0.03 mM NAA	25 μM B, 0.03 mM NAA	100 μM B, 0.03 mM NAA	control
Changes in cell number					
Cambial region	2.8 ±0.45	3.8 ±0.84	3.25 ±0.5	3.4 ±0.55	2.5 ±0.55
RE region	10.6 ±2.416609	14.4 ±1.516575	12.25 ±1.5	12.2 ±2.588436	15 ±2.529822
Developing cells region	7 ±1	8 ±1	7.5 ±0.578	8 ±0.707	6.333 ±0.816
Cambial region cells					
lumen area (μm^2)	181.626 ±60.06	232 ±89.83	163.97 ±97.336	225.217 ±67.343	213.309 ±78.64
radial length (μm)	7.36 ±1.84	8.57 ±2.2	7.304 ±3.062	7.906 ±1.751	7.89 ±2.29
tangential length (μm)	24.4 ±4.05	26.38 ±5.96	22.42 ±6.239	28.633 ±6.663	26.937 ±4.28
RE region cells					
lumen area (μm^2)	428.3777 ±178.0695	482.3839 ±156.1922	500.1149 ±191.9943	557.8588 ±183.6743	587.9382 ±194.3535
radial length (μm)	28.01051 ±5.665497	29.46779 ±6.617918	28.97596 ±6.559155	31.41155 ±5.156675	31.1535 ±5.582775
tangential length (μm)	19.32678 ±4.731532	20.62082 ±3.502307	21.53531 ±4.881192	22.68845 ±4.986826	23.61912 ±4.842087
cell wall area(%μm^2)	31.319	24.136	21.783	28.342	15.861
Existing cells					
lumen area (μm^2)	976.407 ±419.022	632.478 ±266.545	908.391 ±341.005	774.224 ±363.767	906.442 ±480.649
radial length (μm)	41.523 ±9.185	33.994 ±7.277	44.0216 ±7.195	42.921 ±9.388	41.074 ±9.181

tangential length (μm)	29.028 ±8.428	23.208 ±6.469	26.83 ±7.179	23.475 ±7.891	26.475 ±9.205
cell wall area(%μm^2)	21.356	18.986	17.405	18.94	26.57
lignin area in CML/S_1(% lignin μm^2)	3.351	/	/	3.19	3.391
Set No. 4	1 μM B, 0.3 mM NAA	7 μM B, 0.3 mM NAA	25 μM B, 0.3 mM NAA	100 μM B, 0.3 mM NAA	control
culture date 10th Mar 04 Burnham tree					
Growth Period: 2 months					
Changes in cell number					
Cambial region	2.25 ±0.5	3 ±0.82	4 0	3 ±0	2 ±0
RE region	19 2.160247	12 2.160247	18 3.162278	16 1.414214	10 2.345208
Developing cells region	11 0.81649	9.25 0.957427	6 1.224745	5.8 2.48998	11.4 0.547723
Cambial region cells					
lumen area (μm^2)	280.78 ±107.89	317.28 ±112.04	185.94 ±53.83	211.04 ±84.22	288.3 ±71.75
radial length (μm)	11.05 ±3.25	11.38 ±3.19	9.28 ±1.78	10.35 ±2.81	9.25 ±1.87
tangential length (μm)	25.26 ±4.78	27.82 ±4.71	20.02 ±4.17	25.13 ±5.19	31.57 ±6.56
RE region cells					
lumen area (μm^2)	816.7137 ±302.2889	775.913 ±322.2571	666.649 ±301.8079	935.1992 ±371.962	702.6547 ±252.0431
radial length (μm)	36.27936 ±5.732597	35.5267 ±7.384301	36.74867 ±8.477108	38.40798 ±7.018042	34.92903 ±6.247302
tangential length (μm)	28.5287 ±6.782547	27.61191 ±6.347207	23.98015 ±7.077044	30.9761 ±8.053692	26.34547 ±5.957631
cell wall area(%μm^2)	30.78	24.279	31.348	33.485	33.271
Existing cells					
lumen area (μm^2)	1084.513 ±465.97	926.602 ±364.352	1247.652 ±450.376	941.86 ±368.853	1216.329 ±493.763
radial length (μm)	29.136 ±7.411	27.755 ±7.43	32.291 ±7.3904	26.781 ±6.825	30.647 ±8.61
tangential length (μm)	44.61 ±9.416	42.31 ±9.415	49.842 ±12.11	43.244 ±7.694	47.845 ±10.134

cell wall area(%μm^2)	26.448	30.44	36.575	39.734	25.583
lignin area in CML/S_1(% lignin μm^2)	5.721	/	/	3.71	
Set No. 5 culture date 9th Feb 04 Burnham tree Growth Period: 2 months **Changes in cell number**	0 µM B, 0.3 mM NAA	1 µM B, 0.3 mM NAA	7 µM B, 0.3 mM NAA	25 µM B, 0.3 mMNAA	control
Cambial region	2.4 ±0.55	3.75 ±0.5	3.2 0.447	4.75 ±0.5	2 ±0
RE region	12 ±1.871	9 ±1.826	16.2 ±1.303	7.25 ±1.258	15 ±6.481
Developing cells region	2.8 ±0.837	3 ±0.816	3 ±0.707	3.25 ±0.5	2 ±0
Cambial region cells					
lumen area (μm^2)	386.7 ±168.24	327.28 ±148.86	350.61 ±150.13	362.07 ±150.93	337.83 ±151.65
radial length (µm)	12.43 ±4.235	9.24 ±1.863	13.89 ±2.644	13.96 ±2.861	8.8 ±2.51
tangential length (µm)	31.03 ±5.72	31.01 ±6.29	31.036 ±5.59	30.71 ±4.97	28.65 ±6.49
RE region cells					
lumen area (μm^2)	347.3051 ±184.6218	361.0958 ±157.854	324.8983 ±116.339	386.8846 ±139.552	442.5405 ±113.3981
radial length (µm)	24.01289 ±5.543841	26.28006 ±6.618661	24.91653 ±4.838271	26.58651 ±5.59705	30.58722 ±5.805722
tangential length (µm)	17.48874 ±6.5115	17.09269 ±4.36227	15.99487 ±4.461429	17.67388 ±4.223455	17.33663 ±1.971294
cell wall area(%μm^2)	17.984	17.12	20.1	14.276	13.474
Existing cells					
lumen area (μm^2)	1286.918 ±364.988	1120.241 ±394.129	971.968 ±405.981	1000.931 ±400.507	1044.295 ±322.914
radial length (µm)	51.589 ±6.67	47.963 ±8.4876	43.627 ±9.656	43.586 ±7.666	44.396 ±7.686
tangential length (µm)	31.428 ±6.161	28.92 ±6.644	26.989 ±7.558	28.102 ±7.331	29.221 ±5.78
cell wall area(%μm^2)	33.914	18.591	20.288	29.61	25.223

lignin area in CML/S_1(% lignin μm^2)	4.276	4.609	3.946	4.389	1.889
Set No. 6 culture date 25th March 05 Burnham tree Growth Period: 1 month	1 µM B, 0.3 mM NAA	7 µM B, 0.3 mM NAA	25 µM B, 0.3 mMNAA	100, µM B, 0.3 mM NAA	control
Changes in cell number					
Cambial region	4 ±0	4.2 ±0.84	4.6 ±0.55	4.5 ±1.73	2.5 ±0.55
RE region	13.2 ±2.775	13.6 ±2.608	12.6 ±1.95	14 ±1.155	15 ±2.53
Developing cells region	6.4 ±0.548	5.8 ±1.304	6.6 ±0.548	7 ±0	6.333 ±0.816
Cambial region cells					
lumen area (μm^2)	209.618 ±55.809	224.112 ±87.115	272.933 ±76.112	199.33 ±65.228	213.309 ±78.64
radial length (µm)	7.283 ±1.565	8.431 ±2.046	8.758 ±1.963	6.615 ±1.722	7.887 ±2.295
tangential length (µm)	28.978 ±4.981	26.195 ±5.798	31.237 ±5.553	29.908 ±3.2529	26.937 ±4.28
RE region cells					
lumen area (μm^2)	509.1081 ±173.2352	438.101 ±132.1108	531.2199 ±189.2205	468.4962 ±137.1909	±
radial length (µm)	29.26439 ±5.430901	27.94382 ±4.962377	30.6135 ±6.371143	29.2718 ±5.059624	±
tangential length (µm)	21.07978 ±4.418561	19.19416 ±3.21463	21.51048 ±4.338303	20.11981 ±4.042611	±
cell wall area(%μm^2)	40.156	16.95	12.697	19.303	15.861
Existing cells					
lumen area (μm^2)	602.801 ±281.078	690.15 ±329.803	675.308 ±261.47	845.842 ±371.215	906.443 ±480.65
radial length (µm)	32.43 ±7.235	35.022 ±8.358	32.685 ±6.18	37.449 ±8.084	41.074 ±9.181
tangential length (µm)	22.672 ±6.667	24.352 ±6.729	25.071 ±5.765	27.378 ±7.072	26.475 ±9.206
cell wall area(%μm^2)	24.954	24.172	23.12	27.615	26.57
lignin area in CML/S_1(% lignin μm^2)	3.626	/	/	3.912	3.391

Set No. 7 culture date 31st Mar 04 Rotorua tree Growth Period: 3 months	7 µM B, 3 mM NAA	25 µM B, 3 mMNAA	100, µM B, 3 mM NAA	control	
Changes in cell number					
Cambial region	3 ±1	3.33 ±0.52	3.75 ±0.5	2.2 ±0.45	
RE region	15.4 ±1.673	25.5 ±4.087	23.5 ±1.291	22 ±3.391	
Developing cellsregion	12.4 ±1.14	8.667 ±0.516	20.75 ±2.63	15 ±1	
Cambial region cells					
lumen area (μm^2)	264.68 ±73.8	317.71 ±99.83	326.66 ±123.2	326.31 ±99.81	
radial length (µm)	9.69 ±2	10.16 ±2.21	11.46 ±2.62	10.9 ±2.38	
tangential length (µm)	27.18 ±4.53	31.32 ±6.17	31.37 ±4.96	29.56 ±4.66	
RE region cells					
lumen area (μm^2)	837.5971 ±379.3773	722.0557 ±230.9705	843.3124 ±462.6145	940.9401 ±412.8176	
radial length (µm)	37.04566 ±7.770208	34.8397 ±6.036194	36.95268 ±10.59742	39.33993 ±7.510684	
tangential length (µm)	27.87043 ±7.572489	26.19939 ±5.665289	27.83711 ±11.05668	30.17857 ±8.270017	
cell wall area(%μm^2)	33.055	17.046	35.151	42.281	
Existing cells					
lumen area (μm^2)	847.334 ±287.304	583.185 ±266.378	825.275 ±342.183	700.384 ±284.534	
radial length (µm)	38.235 ±6.112	31.809 ±7.121	37.025 ±8.047	37.12 ±6.566	
tangential length (µm)	27.229 ±6.115	22.286 ±5.712	27.119 ±7.222	22.993 ±6.312	
cell wall area(%μm^2)	25.406	19.249	23.206	36.726	
lignin area in CML/S_1(% lignin μm^2)	2.7487		3.288	3.754	

Set No. 8	1 µM B, 3 mM NAA	7 µM B, 3 mM NAA	25 µM B, 3 mMNAA	100, µM B, 3 mM NAA	control
culture date 26th Apr 04 Rotorua tree 3					
Growth Period: 1 month					
Changes in cell number					
Cambial region	4 ±1	3 ±0	3 ±0	3.5 ±0.58	2 ±0
RE region	25.8 ±1.924	19.4 ±2.881	26.5 ±1.291	24 ±2.943	25.167 ±2.563
Developing cellsregion	20.2 ±3.347	17.8 ±1.309	19.75 ±1.5	17 ±2.161	14 ±1.414
Cambial region cells					
lumen area (μm^2)	321.87 ±149.57	304.07 ±155.53	282.13 ±156.74	278.02 ±151.9	208.35 ±68.93
radial length (µm)	9.91 ±1.1	6.12 ±1.19	6.43 ±1.43	9.28 ±1.66	7.16 ±2.14
tangential length (µm)	25.15 ±6.1	17.38 ±4.26	21.31 ±3.96	27.82 ±6.51	29.18 ±4.85
RE region cells					
lumen area (μm^2)	751.048 ±251.8525	709.1751 ±295.9416	495.706 ±196.392	482.3725 ±221.4232	564.6569 ±253.7051
radial length (µm)	34.55151 ±5.110121	36.84056 ±8.600698	30.04507 ±5.997459	28.2458 ±6.605405	31.56085 ±7.387155
tangential length (µm)	27.41221 ±5.912188	24.25687 ±5.661075	21.07344 ±5.40339	21.7343 ±4.963922	22.2939 ±5.819177
cell wall area(%μm^2)	6.923	14.305	17.296	11.478	16.692
Existing cells					
lumen area (μm^2)	584.575 ±258.664	757.029 ±289.094	455.359 ±217.442	687.054 ±332.552	678.536 ±264.266
radial length (µm)	31.524 ±7.987	35.867 ±6.19	27.629 ±6.663	34.538 ±9.308	36 ±8.64
tangential length (µm)	22.334 ±6.8	27.954 ±6.71	20.292 ±6.81	24.753 ±7.565	23.83 ±6.778
cell wall area(%μm^2)	23.80516	26.958	15.331	15.076	14.347
lignin area in CML/S_1(% lignin μm^2)	5.607521	4.316923	2.757904	4.335009	3.622167

Set No. 9 culture date 25th March 05 Burnham tree Growth Period: 1 month	1 µM B, 3 mM NAA	7 µM B, 3 mM NAA	25 µM B, 3 mMNAA	100, µM B, 3 mM NAA	control
Changes in cell number					
Cambial region	3.6 ±0.55	4.2 ±0.45	3.2 ±0.45	4.67 ±0.52	2.5 ±0.55
RE region	12.4 ±1.817	16 ±2	11 ±0.707	12.167 ±1.472	15 ±2.53
Developing cellsregion	5.4 ±0.548	6.4 ±1.14	4.8 ±0.447	8 ±0.894	6.333 ±0.816
Cambial region cells					
lumen area (μm^2)	216.276 ±85.7657	180.793 ±68.05	170.819 ±62.898	223.183 ±61.516	213.309 ±78.64
radial length (µm)	7.799 ±2.275	6.62 ±1.79	7.408 ±1.93	8.1943 ±1.966	7.887 ±2.295
tangential length (µm)	27.417 ±4.647	27.012 ±6.667	22.904 ±4.757	27.367 ±5.024	26.937 ±4.28
RE region cells					
lumen area (μm^2)	551.1207 ±179.9747	602.923 ±240.8522	529.9447 180.0658	516.6298 181.3331	/
radial length (µm)	29.63549 ±4.625766	31.44967 6.81440	30.68647 6.0182	28.53087 5.44573	/
tangential length (µm)	23.2772 ±4.811631	24.01695 ±5.468623	21.86105 4.692211	23.0519 4.653226	/
cell wall area(%μm^2)	25.146	24.779	20.196	21.018	15.861
Existing cells					
lumen area (μm^2)	911.225 ±431.187	745.402 ±311.255	990.942 ±430.762	904.533 ±347.466	906.4427 ±480.649
radial length (µm)	41.435 ±11.108	34.881 ±7.847	40.986 ±9.241	38.93 ±7.364	41.074 ±9.181
tangential length (µm)	26.967 ±8.418	26.457 ±6.961	29.237 ±7.114	28.685 ±6.458	26.475 ±9.205
cell wall area(%μm^2)	37.022	29.724	17.412	28.777	26.57
lignin area in CML/S_1(% lignin μm^2)	6.893	/	/	3.286	3.391

Statistical analysis for data presented in chapter seven

For the ease of the analysis the treatments were coded with number from 1 to 13.

Code number 1 – 1 µM B, 0.3 mM NAA
Code number 2 – 7 µM B, 0.3 mM NAA
Code number 3 – 25 µM B, 0.3 mM NAA
Code number 4 – 100 µM B, 0.3 mM NAA
Code number 5 – control
Code number 6 – 1 µM B, 3 mM NAA
Code number 7 – 7 µM B, 3 mM NAA
Code number 8 – 25 µM B, 3 mM NAA
Code number 9 – 100 µM B, 3 mM NAA
Code number 10 – 1 µM B, 0.3 mM NAA
Code number 11 – 7 µM B, 0.3 mM NAA
Code number 12 – 25 µM B, 0.3 mM NAA
Code number 13 – 100 µM B, 0.3 mM NAA

Auxin and boron treated cambial cell count analysis

Completely Randomized AOV for cambial

Source	DF	SS	MS	F	P
Codes	12	91.141	7.59505	12.5	0.0000
Error	183	111.242	0.60788		
Total	195	202.383			

Grand Mean 3.4439 CV 22.64

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	46.6	12	0.0000
Cochran's Q	0.2460		
Largest Var / Smallest Var	14.248		

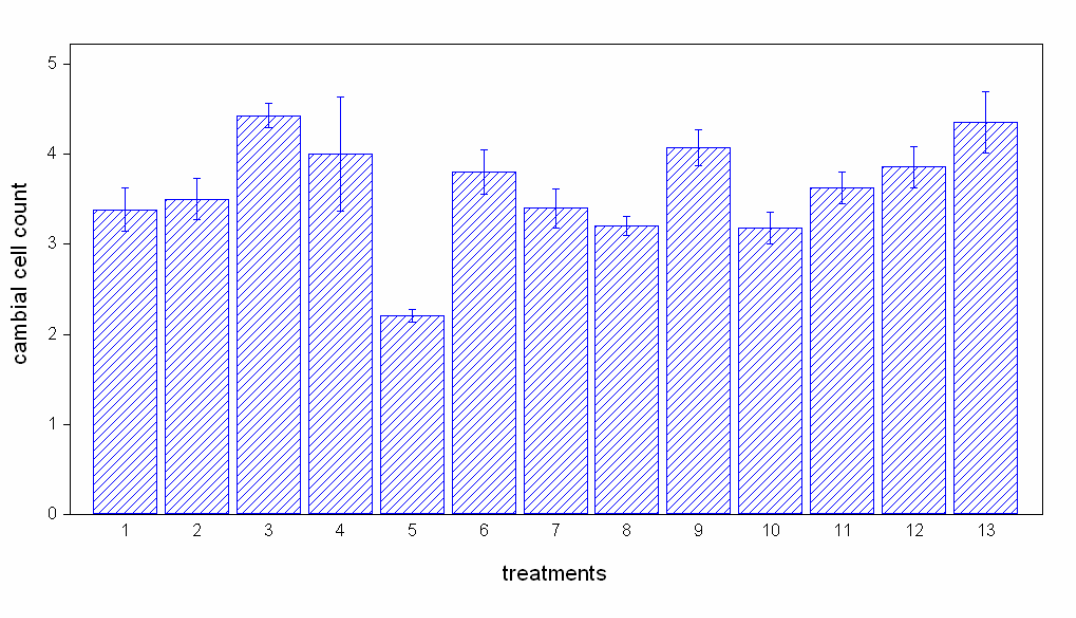
Component of variance for between groups 0.46981
Effective cell size 14.9

codes	N	Mean	SE	codes	N	Mean	SE
1	13	3.3846	0.2162	8	15	3.2000	0.2013
2	14	3.5000	0.2084	9	14	4.0714	0.2084
3	14	4.4286	0.2084	10	17	3.1765	0.1891
4	6	4.0000	0.3183	11	16	3.6250	0.1949
5	34	2.2059	0.1337	12	14	3.8571	0.2084
6	10	3.8000	0.2466	13	14	4.3571	0.2084
7	15	3.4000	0.2013				

Tukey HSD All-Pairwise Comparisons Test of cambial by codes

codes	Mean	Homogeneous Groups
3	4.4286	A
13	4.3571	AB
9	4.0714	ABC
4	4.0000	ABC
12	3.8571	ABC
6	3.8000	ABC
11	3.6250	ABC
2	3.5000	ABC
7	3.4000	BC
1	3.3846	BC
8	3.2000	C
10	3.1765	C
5	2.2059	D

Alpha 0.05
Critical Q Value 4.679
There are 4 groups (A, B, etc.) in which the means
are not significantly different from one another.



Auxin and boron treated RE cell count analysis

Completely Randomized AOV for RE

Source	DF	SS	MS	F	P
codes	12	920.17	76.6805	2.29	0.0098
Error	183	6131.32	33.5045		
Total	195	7051.49			

Grand Mean 16.949 CV 34.15

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	42.4	12	0.0000
Cochran's Q	0.1521		
Largest Var / Smallest Var	28.063		

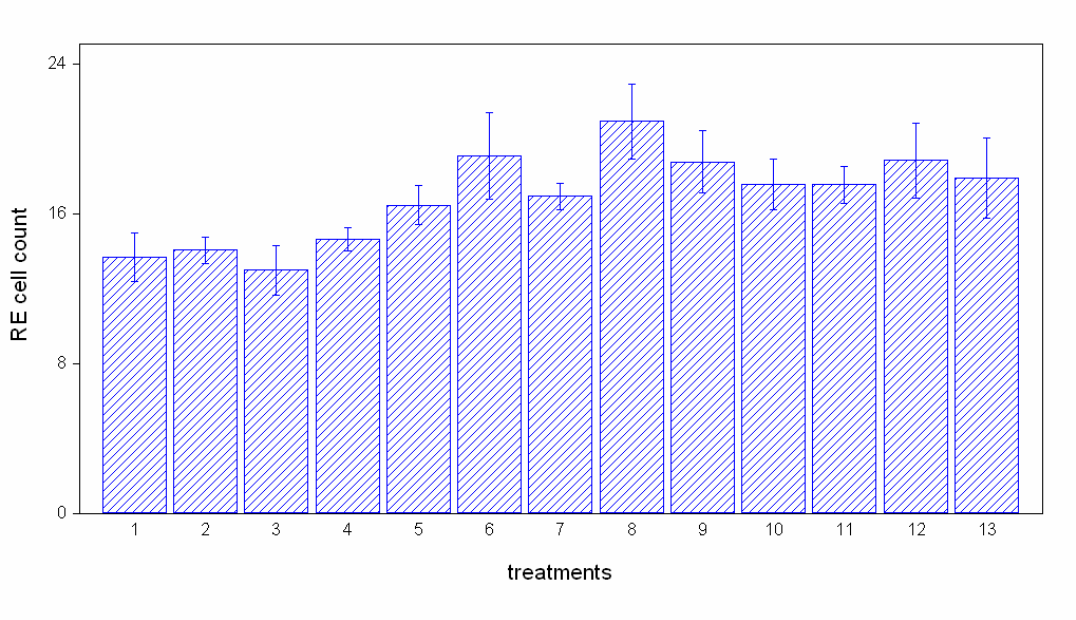
Component of variance for between groups 2.90309
Effective cell size 14.9

codes	N	Mean	SE	codes	N	Mean	SE
1	13	13.692	1.6054	8	15	20.933	1.4945
2	14	14.071	1.5470	9	14	18.786	1.5470
3	14	13.000	1.5470	10	17	17.588	1.4039
4	6	14.667	2.3631	11	16	17.563	1.4471
5	34	16.471	0.9927	12	14	18.857	1.5470
6	10	19.100	1.8304	13	14	17.929	1.5470
7	15	16.933	1.4945				

Tukey HSD All-Pairwise Comparisons Test of RE by codes

codes	Mean	Homogeneous Groups
8	20.933	A
6	19.100	AB
12	18.857	AB
9	18.786	AB
13	17.929	AB
10	17.588	AB
11	17.563	AB
7	16.933	AB
5	16.471	AB
4	14.667	AB
2	14.071	AB
1	13.692	AB
3	13.000	B

Alpha 0.05
Critical Q Value 4.679
There are 2 groups (A and B) in which the means
are not significantly different from one another.



Auxin and boron treated developing cell count analysis

Completely Randomized AOV for new

Source	**DF**	**SS**	**MS**	**F**	**P**
codes	12	1956.34	163.028	7.23	0.0000
Error	182	4104.53	22.552		
Total	194	6060.87			

Grand Mean 11.026 CV 43.07

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	45.1	12	0.0000
Cochran's Q	0.2240		
Largest Var / Smallest Var	37.333		

Component of variance for between groups 9.48505
Effective cell size 14.8

codes	**N**	**Mean**	**SE**	**codes**	**N**	**Mean**	**SE**
1	13	6.769	1.3171	8	15	10.333	1.2262
2	14	5.786	1.2692	9	14	14.214	1.2692
3	14	5.429	1.2692	10	17	12.176	1.1518
4	6	6.167	1.9387	11	16	15.750	1.1872
5	33	11.121	0.8267	12	14	12.929	1.2692
6	10	12.800	1.5017	13	14	14.000	1.2692
7	15	12.200	1.2262				

Tukey HSD All-Pairwise Comparisons Test of new by codes

codes	Mean	1	2	3	4	5	6	7
1	6.7692							
2	5.7857	0.984						
3	5.4286	1.341	0.357					
4	6.1667	0.603	0.381	0.738				
5	11.121	4.352	5.335*	5.693*	4.955			
6	12.800	6.031	7.014*	7.371*	6.633	1.679		
7	12.200	5.431	6.414*	6.771*	6.033	1.079	0.600	
8	10.333	3.564	4.548	4.905	4.167	0.788	2.467	1.867
9	14.214	7.445*	8.429*	8.786*	8.048*	3.093	1.414	2.014
10	12.176	5.407	6.391*	6.748*	6.010	1.055	0.624	0.024
11	15.750	8.981*	9.964*	10.321*	9.583*	4.629	2.950	3.550
12	12.929	6.159*	7.143*	7.500*	6.762	1.807	0.129	0.729
13	14.000	7.231*	8.214*	8.571*	7.833*	2.879	1.200	1.800

codes	Mean	8	9	10	11	12
8	10.333					
9	14.214	3.881				
10	12.176	1.843	2.038			
11	15.750	5.417	1.536	3.574		
12	12.929	2.595	1.286	0.752	2.821	
13	14.000	3.667	0.214	1.824	1.750	1.071

Alpha 0.05
Critical Q Value 4.679
The homogeneous group format can't be used
because of the pattern of significant differences.

Auxin and boron treated cambial cell lumen area analysis

Completely Randomized AOV for area

Source	DF	SS	MS	F	P
sample	12	1009420	84118.3	7.38	0.0000
Error	737	8403890	11402.8		
Total	749	9413310			

Grand Mean 251.76 CV 42.42

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	33.7	12	0.0008

Cochran's Q 0.1256
Largest Var / Smallest Var 3.2123

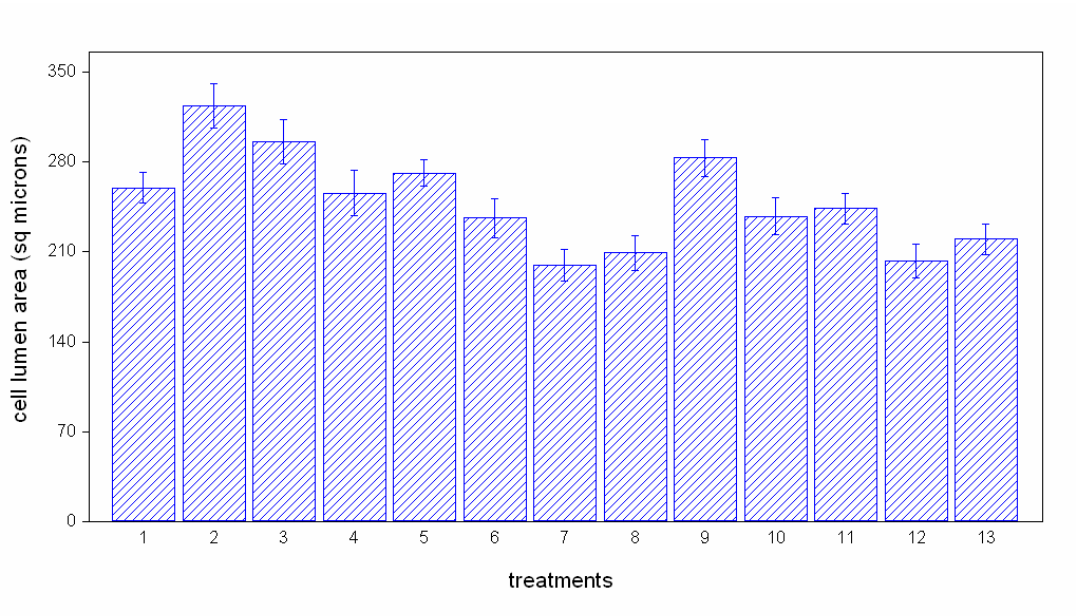
Component of variance for between groups 1275.21
Effective cell size 57.0

sample	N	Mean	SE	sample	N	Mean	SE
1	60	259.87	13.786	8	60	209.22	13.786
2	60	323.65	13.786	9	60	283.18	13.786
3	60	296.02	13.786	10	60	237.57	13.786
4	20	255.81	23.878	11	40	243.65	16.884
5	120	271.45	9.748	12	60	203.02	13.786
6	40	236.29	16.884	13	60	219.83	13.786
7	50	199.77	15.102				

Tukey HSD All-Pairwise Comparisons Test of area by sample

sample	Mean	Homogeneous Groups
2	323.65	A
3	296.02	AB
9	283.18	ABC
5	271.45	ABC
1	259.87	ABCD
4	255.81	ABCD
11	243.65	BCD
10	237.57	BCD
6	236.29	BCD
13	219.83	CD
8	209.22	D
12	203.02	D
7	199.77	D

Alpha 0.05
Critical Q Value 4.679
There are 4 groups (A, B, etc.) in which the means
are not significantly different from one another.



Auxin and boron treated cambial radial length analysis

Completely Randomized AOV for radial

Source	DF	SS	MS	F	P
sample	12	851.44	70.9531	9.95	0.0000
Error	737	5256.01	7.1316		
Total	749	6107.44			

Grand Mean 9.0414 CV 29.54

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	37.8	12	0.0002
Cochran's Q	0.1323		
Largest Var / Smallest Var	3.9515		

Component of variance for between groups 1.11924
Effective cell size 57.0

sample	N	Mean	SE	sample	N	Mean	SE
1	60	9.193	0.3448	8	60	7.998	0.3448
2	60	11.232	0.3448	9	60	9.642	0.3448
3	60	10.666	0.3448	10	60	8.453	0.3448
4	20	10.352	0.5971	11	40	9.009	0.4222
5	120	9.151	0.2438	12	60	8.105	0.3448
6	40	8.853	0.4222	13	60	7.613	0.3448
7	50	7.746	0.3777				

Tukey HSD All-Pairwise Comparisons Test of radial by sample

sample	Mean	1	2	3	4	5	6	7
1	9.1931							
2	11.232	2.039*						
3	10.666	1.473	0.566					
4	10.352	1.159	0.880	0.314				
5	9.1506	0.042	2.081*	1.515*	1.201			
6	8.8533	0.340	2.378*	1.813*	1.498	0.297		
7	7.7463	1.447	3.485*	2.920*	2.605*	1.404	1.107	
8	7.9981	1.195	3.234*	2.668*	2.354*	1.153	0.855	0.252
9	9.6421	0.449	1.590	1.024	0.710	0.491	0.789	1.896*
10	8.4528	0.740	2.779*	2.213*	1.899	0.698	0.401	0.706
11	9.0087	0.184	2.223*	1.657	1.343	0.142	0.155	1.262
12	8.1047	1.088	3.127*	2.561*	2.247	1.046	0.749	0.358
13	7.6133	1.580	3.618*	3.053*	2.738*	1.537*	1.240	0.133

sample	Mean	8	9	10	11	12
8	7.9981					
9	9.6421	1.644*				
10	8.4528	0.455	1.189			
11	9.0087	1.011	0.633	0.556		
12	8.1047	0.107	1.537	0.348	0.904	
13	7.6133	0.385	2.029*	0.839	1.395	0.491

Alpha 0.05
Critical Q Value 4.679
The homogeneous group format can't be used
because of the pattern of significant differences.

Auxin and boron treated cambial tangential length analysis

Completely Randomized AOV for tangential length

Source	**DF**	**SS**	**MS**	**F**	**P**
sample	12	1915.3	159.605	4.21	0.0000
Error	737	27963.7	37.943		
Total	749	29878.9			

Grand Mean 27.288 CV 22.57

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	13.3	12	0.3508

Cochran's Q 0.1037
Largest Var / Smallest Var 1.8812

Component of variance for between groups 2.13359
Effective cell size 57.0

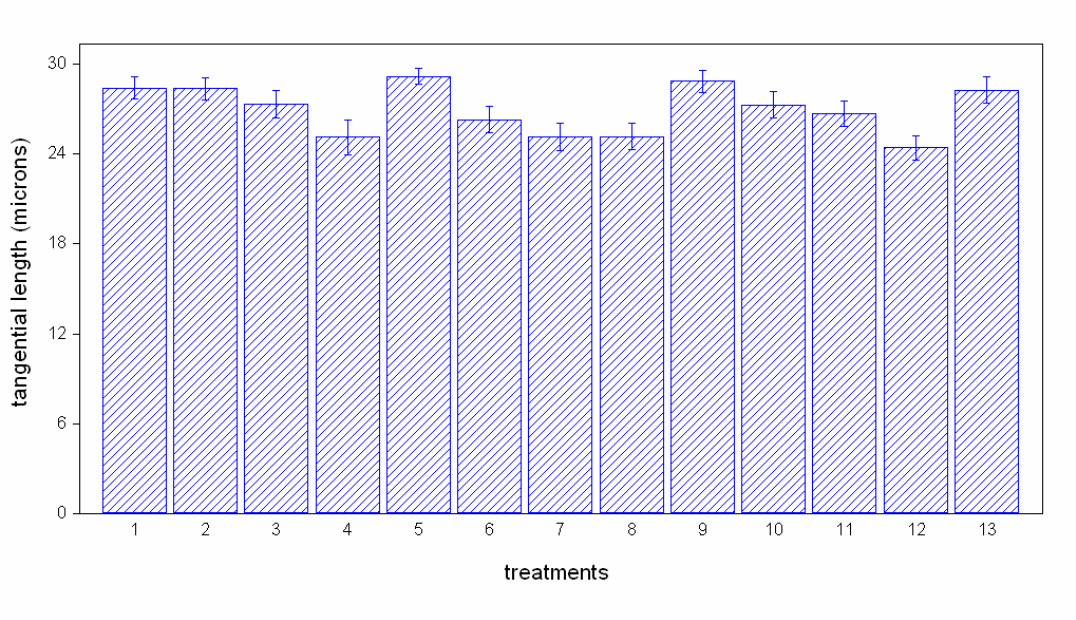
sample	N	Mean	SE	sample	N	Mean	SE
1	60	28.414	0.7952	8	60	25.176	0.7952
2	60	28.350	0.7952	9	60	28.853	0.7952
3	60	27.323	0.7952	10	60	27.289	0.7952
4	20	25.125	1.3774	11	40	26.698	0.9739
5	120	29.178	0.5623	12	60	24.415	0.7952
6	40	26.286	0.9739	13	60	28.264	0.7952
7	50	25.152	0.8711				

Tukey HSD All-Pairwise Comparisons Test of tangentia by sample

sample	Mean	1	2	3	4	5	6	7
1	28.414							
2	28.350	0.064						
3	27.323	1.091	1.027					
4	25.125	3.289	3.225	2.197				
5	29.178	0.764	0.828	1.855	4.053			
6	26.286	2.128	2.065	1.037	1.160	2.893		
7	25.152	3.262	3.198	2.171	0.027	4.026*	1.134	
8	25.176	3.238	3.174	2.147	0.050	4.002*	1.110	0.024
9	28.853	0.439	0.503	1.530	3.728	0.325	2.567	3.701
10	27.289	1.125	1.062	0.034	2.163	1.890	1.003	2.137
11	26.698	1.716	1.652	0.624	1.573	2.480	0.413	1.546
12	24.415	3.999*	3.935*	2.907	0.710	4.763*	1.870	0.737
13	28.264	0.150	0.087	0.941	3.138	0.915	1.978	3.112

sample	Mean	8	9	10	11	12
8	25.176					
9	28.853	3.677				
10	27.289	2.113	1.564			
11	26.698	1.523	2.155	0.590		
12	24.415	0.761	4.438*	2.873	2.283	
13	28.264	3.088	0.589	0.975	1.565	3.848*

Alpha 0.05
Critical Q Value 4.679
The homogeneous group format can't be used
because of the pattern of significant differences.



Auxin and boron treated developing cells cell lumen area analysis

Completely Randomized AOV for area

Source	**DF**	**SS**	**MS**	**F**	**P**
sample	12	1139131	94927.6	8.46	0.0000
Error	737	8274179	11226.8		
Total	749	9413310			

Grand Mean 251.76 CV 42.09

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	41.4	12	0.0000
Cochran's Q	0.1294		
Largest Var / Smallest Var	3.7235		

Component of variance for between groups 1469.01
Effective cell size 57.0

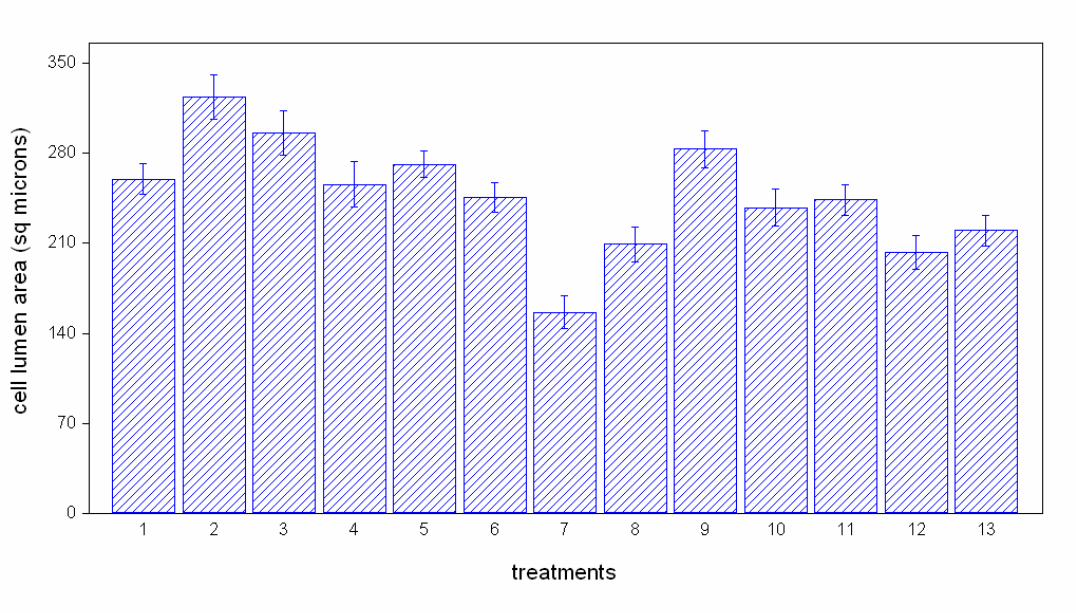
sample	**N**	**Mean**	**SE**	**sample**	**N**	**Mean**	**SE**
1	60	259.87	13.679	8	60	209.22	13.679
2	60	323.65	13.679	9	60	283.18	13.679
3	60	296.02	13.679	10	60	237.57	13.679
4	20	255.81	23.693	11	40	243.65	16.753
5	120	271.45	9.672	12	60	203.02	13.679
6	60	245.75	13.679	13	60	219.83	13.679
7	30	156.49	19.345				

Tukey HSD All-Pairwise Comparisons Test of area by sample

sample	Mean	1	2	3	4	5	6	7
1	259.87							
2	323.65	63.79						
3	296.02	36.15	27.64					
4	255.81	4.06	67.85	40.21				
5	271.45	11.58	52.21	24.57	15.64			
6	245.75	14.12	77.90*	50.27	10.06	25.70		
7	156.49	103.38*	167.16*	139.53*	99.32	114.96*	89.26*	
8	209.22	50.65	114.43*	86.79*	46.58	62.23*	36.53	52.73
9	283.18	23.32	40.47	12.83	27.38	11.74	37.43	126.70*
10	237.57	22.30	86.08*	58.44	18.23	33.88	8.18	81.08*
11	243.65	16.21	80.00*	52.36	12.15	27.80	2.10	87.16*
12	203.02	56.85	120.64*	93.00*	52.79	68.43*	42.73	46.53
13	219.83	40.03	103.82*	76.18*	35.97	51.61	25.92	63.34

sample	Mean	8	9	10	11	12
8	209.22					
9	283.18	73.96*				
10	237.57	28.35	45.61			
11	243.65	34.43	39.53	6.08		
12	203.02	6.20	80.17*	34.55	40.64	
13	219.83	10.61	63.35	17.74	23.82	16.82

Alpha 0.05
Critical Q Value 4.679
The homogeneous group format can't be used
because of the pattern of significant differences.



Auxin and boron treated developing cells radial length analysis

Completely Randomized AOV for radial

Source	DF	SS	MS	F	P
sample	12	967.49	80.6245	11.6	0.0000
Error	737	5139.95	6.9742		
Total	749	6107.44			

Grand Mean 9.0414 CV 29.21

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	49.0	12	0.0000
Cochran's Q	0.1376		
Largest Var / Smallest Var	4.6108		

Component of variance for between groups 1.29262
Effective cell size 57.0

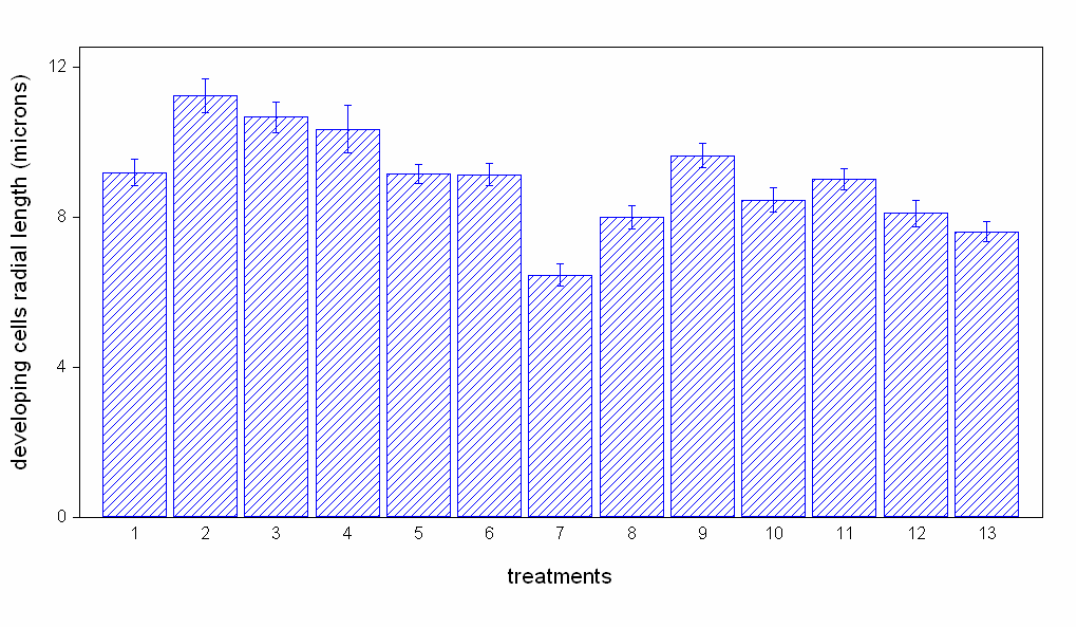
sample	N	Mean	SE	sample	N	Mean	SE
1	60	9.193	0.3409	8	60	7.998	0.3409
2	60	11.232	0.3409	9	60	9.642	0.3409
3	60	10.666	0.3409	10	60	8.453	0.3409
4	20	10.352	0.5905	11	40	9.009	0.4176
5	120	9.151	0.2411	12	60	8.105	0.3409
6	60	9.131	0.3409	13	60	7.613	0.3409
7	30	6.454	0.4822				

Tukey HSD All-Pairwise Comparisons Test of radial by sample

sample	Mean	1	2	3	4	5	6	7
1	9.1931							
2	11.232	2.039*						
3	10.666	1.473	0.566					
4	10.352	1.159	0.880	0.314				
5	9.1506	0.042	2.081*	1.515*	1.201			
6	9.1305	0.063	2.101*	1.535	1.221	0.020		
7	6.4539	2.739*	4.778*	4.212*	3.898*	2.697*	2.677*	
8	7.9981	1.195	3.234*	2.668*	2.354*	1.153	1.132	1.544
9	9.6421	0.449	1.590	1.024	0.710	0.491	0.512	3.188*
10	8.4528	0.740	2.779*	2.213*	1.899	0.698	0.678	1.999*
11	9.0087	0.184	2.223*	1.657	1.343	0.142	0.122	2.555*
12	8.1047	1.088	3.127*	2.561*	2.247	1.046	1.026	1.651
13	7.6133	1.580	3.618*	3.053*	2.738*	1.537*	1.517	1.159

sample	Mean	8	9	10	11	12
8	7.9981					
9	9.6421	1.644*				
10	8.4528	0.455	1.189			
11	9.0087	1.011	0.633	0.556		
12	8.1047	0.107	1.537	0.348	0.904	
13	7.6133	0.385	2.029*	0.839	1.395	0.491

Alpha 0.05
Critical Q Value 4.679
The homogeneous group format can't be used
because of the pattern of significant differences.



Auxin and boron treated developing cells tangential length analysis

Completely Randomized AOV for tangential length

Source	**DF**	**SS**	**MS**	**F**	**P**
sample	12	2041.5	170.129	4.50	0.0000
Error	737	27837.4	37.771		
Total	749	29878.9			

Grand Mean 27.288 CV 22.52

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	17.5	12	0.1316
Cochran's Q	0.1128		
Largest Var / Smallest Var	2.1080		

Component of variance for between groups 2.32297
Effective cell size 57.0

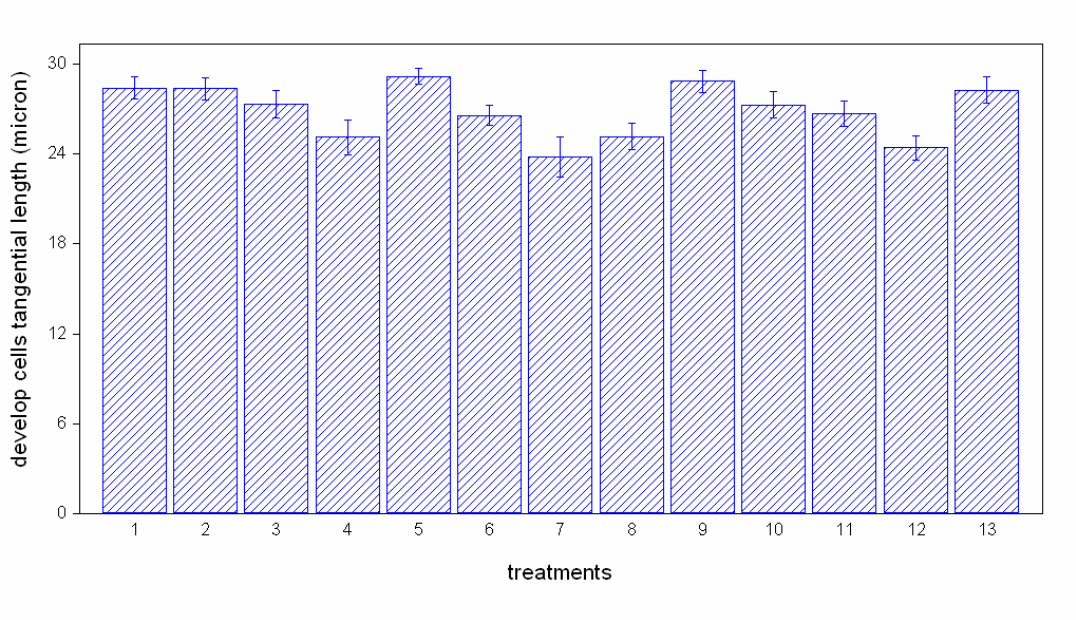
sample	N	Mean	SE	sample	N	Mean	SE
1	60	28.414	0.7934	8	60	25.176	0.7934
2	60	28.350	0.7934	9	60	28.853	0.7934
3	60	27.323	0.7934	10	60	27.289	0.7934
4	20	25.125	1.3742	11	40	26.698	0.9717
5	120	29.178	0.5610	12	60	24.415	0.7934
6	60	26.583	0.7934	13	60	28.264	0.7934
7	30	23.801	1.1221				

Tukey HSD All-Pairwise Comparisons Test of tangentia by sample

sample	Mean	1	2	3	4	5	6	7
1	28.414							
2	28.350	0.064						
3	27.323	1.091	1.027					
4	25.125	3.289	3.225	2.197				
5	29.178	0.764	0.828	1.855	4.053			
6	26.583	1.831	1.767	0.739	1.458	2.595		
7	23.801	4.613*	4.549*	3.522	1.324	5.377*	2.783	
8	25.176	3.238	3.174	2.147	0.050	4.002*	1.408	1.375
9	28.853	0.439	0.503	1.530	3.728	0.325	2.270	5.052*
10	27.289	1.125	1.062	0.034	2.163	1.890	0.705	3.488
11	26.698	1.716	1.652	0.624	1.573	2.480	0.115	2.898
12	24.415	3.999*	3.935*	2.907	0.710	4.763*	2.168	0.614
13	28.264	0.150	0.087	0.941	3.138	0.915	1.680	4.463

sample	Mean	8	9	10	11	12
8	25.176					
9	28.853	3.677				
10	27.289	2.113	1.564			
11	26.698	1.523	2.155	0.590		
12	24.415	0.761	4.438*	2.873	2.283	
13	28.264	3.088	0.589	0.975	1.565	3.848*

Alpha 0.05
Critical Q Value 4.679
The homogeneous group format can't be used
because of the pattern of significant differences.



Auxin and boron treated existing cells cell lumen area analysis

Completely Randomized AOV for cell

Source	**DF**	**SS**	**MS**	**F**	**P**
sample	12	3.429E+07	2857398	20.9	0.0006
Error	4427	6.055E+08	136763		
Total	4439	6.397E+08			

Grand Mean 803.67 CV 46.02

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	121	12	0.0000
Cochran's Q	0.1150		
Largest Var / Smallest Var	2.2484		

Component of variance for between groups 8066.14
Effective cell size 337.3

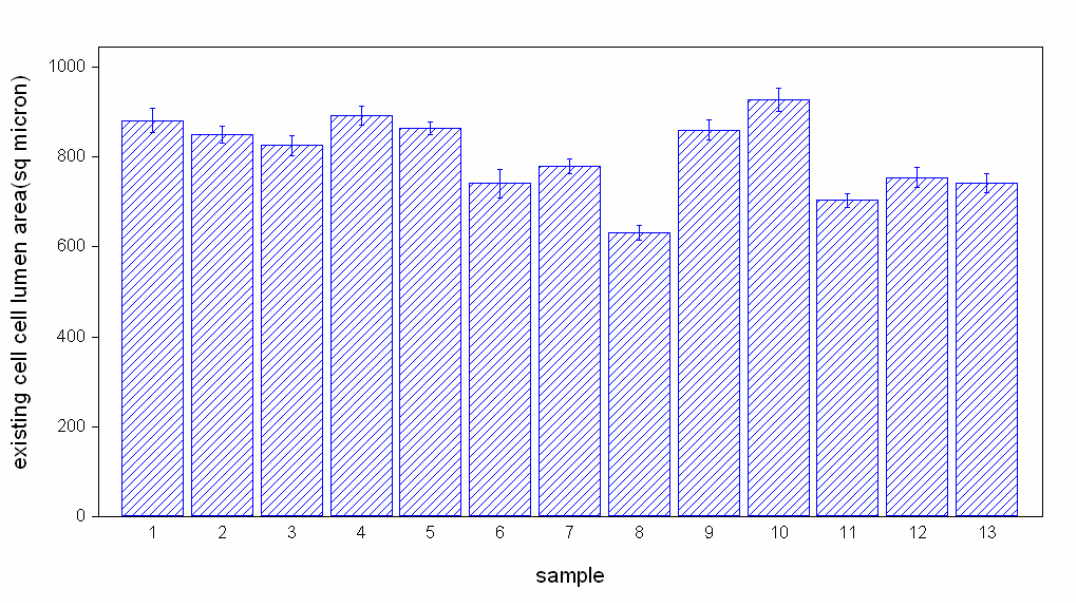
sample	**N**	**Mean**	**SE**	**sample**	**N**	**Mean**	**SE**
1	281	881.03	22.061	8	492	631.38	16.673
2	447	849.24	17.492	9	256	860.26	23.113
3	310	825.33	21.004	10	325	926.82	20.514
4	291	892.04	21.679	11	422	703.09	18.002
5	671	864.25	14.277	12	238	754.39	23.972
6	148	741.28	30.399	13	221	742.15	24.876
7	338	780.24	20.115				

Tukey HSD All-Pairwise Comparisons Test of cell by sample

sample	Mean	1	2	3	4	5	6	7
1	881.03							
2	849.24	31.79						
3	825.33	55.70	23.91					
4	892.04	11.01	42.80	66.71				
5	864.25	16.78	15.01	38.92	27.78			
6	741.28	139.75*	107.96	84.05	150.76*	122.97*		
7	780.24	100.79*	69.00	45.09	111.79*	84.01*	38.96	
8	631.38	249.65*	217.86*	193.95*	260.66*	232.87*	109.90	148.86*
9	860.26	20.77	11.02	34.93	31.78	3.99	118.98	80.02
10	926.82	45.79	77.58	101.49*	34.78	62.56	185.54*	146.58*
11	703.09	177.94*	146.15*	122.24*	188.95*	161.16*	38.19	77.15
12	754.39	126.64*	94.85	70.94	137.65*	109.86*	13.11	25.85
13	742.15	138.89*	107.09*	83.18	149.89*	122.11*	0.87	38.10

sample	Mean	8	9	10	11	12
8	631.38					
9	860.26	228.88*				
10	926.82	295.44*	66.56			
11	703.09	71.71	157.17*	223.73*		
12	754.39	123.01*	105.87	172.43*	51.30	
13	742.15	110.77*	118.11*	184.67*	39.05	12.24

Alpha 0.05
Critical Q Value 4.679
The homogeneous group format can't be used
because of the pattern of significant differences.



Auxin and boron treated existing cells radial length analysis

Completely Randomized AOV for radial

Source	DF	SS	MS	F	P
sample	12	8857	738.080	14.0	0.0000
Error	4427	233363	52.713		
Total	4439	242220			

Grand Mean 25.962 CV 27.97

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	42.0	12	0.0000

Cochran's Q 0.1109
Largest Var / Smallest Var 1.7195

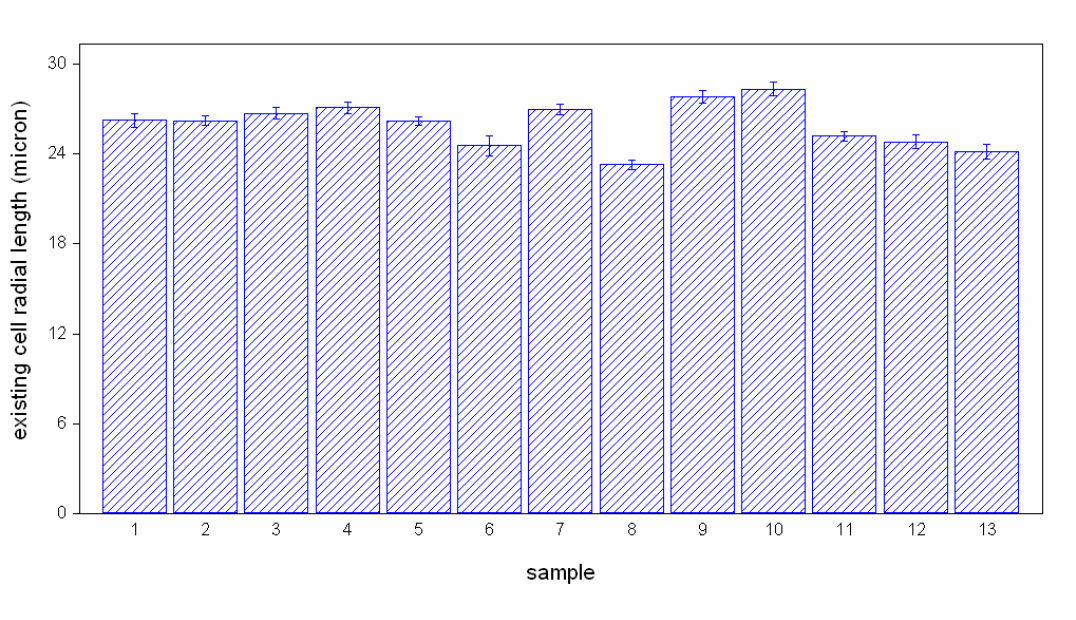
Component of variance for between groups 2.03197
Effective cell size 337.3

sample	N	Mean	SE	sample	N	Mean	SE
1	281	26.262	0.4331	8	492	23.278	0.3273
2	447	26.232	0.3434	9	256	27.810	0.4538
3	310	26.724	0.4124	10	325	28.339	0.4027
4	291	27.091	0.4256	11	422	25.181	0.3534
5	671	26.213	0.2803	12	238	24.816	0.4706
6	148	24.556	0.5968	13	221	24.146	0.4884
7	338	26.996	0.3949				

Tukey HSD All-Pairwise Comparisons Test of radial by sample

sample	Mean	Homogeneous Groups
10	28.339	A
9	27.810	AB
4	27.091	AB
7	26.996	AB
3	26.724	ABC
1	26.262	BCD
2	26.232	BCD
5	26.213	BCD
11	25.181	CD
12	24.816	CDE
6	24.556	CDE
13	24.146	DE
8	23.278	E

Alpha 0.05
Critical Q Value 4.679
There are 5 groups (A, B, etc.) in which the means
are not significantly different from one another.



Auxin and boron treated existing cells tangential length analysis

Completely Randomized AOV for tangetial

Source	**DF**	**SS**	**MS**	**F**	**P**
sample	12	29292	2441.02	30.8	0.0006
Error	4427	350978	79.28		
Total	4439	380270			

Grand Mean 37.776 CV 23.57

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	143	12	0.0000
Cochran's Q	0.1093		
Largest Var / Smallest Var	2.2721		

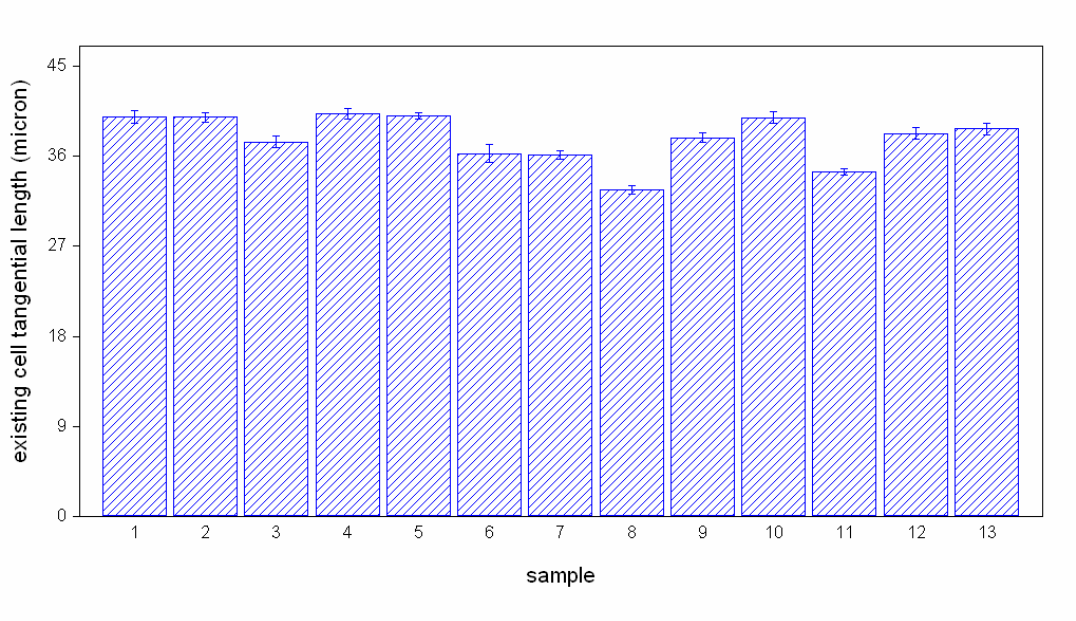
Component of variance for between groups 7.00209
Effective cell size 337.3

sample	**N**	**Mean**	**SE**	**sample**	**N**	**Mean**	**SE**
1	281	39.971	0.5312	8	492	32.617	0.4014
2	447	39.900	0.4211	9	256	37.866	0.5565
3	310	37.436	0.5057	10	325	39.853	0.4939
4	291	40.237	0.5220	11	422	34.438	0.4334
5	671	40.039	0.3437	12	238	38.255	0.5772
6	148	36.279	0.7319	13	221	38.740	0.5989
7	338	36.145	0.4843				

Tukey HSD All-Pairwise Comparisons Test of tangetial by sample

sample	Mean	Homogeneous Groups
4	40.237	A
5	40.039	A
1	39.971	A
2	39.900	A
10	39.853	A
13	38.740	AB
12	38.255	ABC
9	37.866	ABC
3	37.436	BC
6	36.279	BCD
7	36.145	CD
11	34.438	DE
8	32.617	E

Alpha 0.05
Critical Q Value 4.679
There are 5 groups (A, B, etc.) in which the means
are not significantly different from one another.



Auxin and boron treated developing cells % cell wall thickness

Completely Randomized AOV for C

Source	DF	SS	MS	F	P
samples	12	739.05	61.588	0.61	0.8153
Error	27	2728.08	101.040		
Total	39	3467.14			

Grand Mean 24.051 CV 41.79

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	15.9	12	0.1978

Cochran's Q 0.2016
Largest Var / Smallest Var 75.570

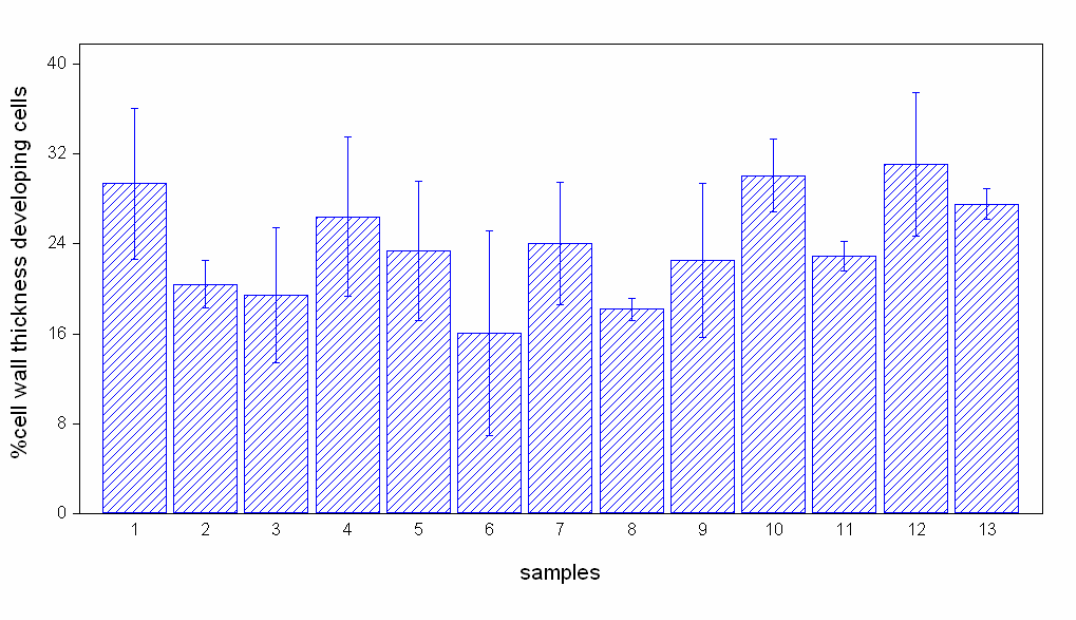
Component of variance for between groups -12.9175
Effective cell size 3.1

samples	N	Mean	SE	samples	N	Mean	SE
1	3	29.352	5.8035	8	3	18.179	5.8035
2	3	20.409	5.8035	9	3	22.549	5.8035
3	3	19.440	5.8035	10	3	30.092	5.8035
4	2	26.394	7.1077	11	3	22.903	5.8035
5	6	23.393	4.1037	12	3	31.101	5.8035
6	2	16.035	7.1077	13	3	27.532	5.8035
7	3	24.046	5.8035				

Tukey HSD All-Pairwise Comparisons Test of C by samples

samples	Mean	Homogeneous Groups
12	31.101	A
10	30.092	A
1	29.352	A
13	27.532	A
4	26.394	A
7	24.046	A
5	23.393	A
11	22.903	A
9	22.549	A
2	20.409	A
3	19.440	A
8	18.179	A
6	16.035	A

Alpha 0.05
Critical Q Value 5.120
There are no significant pairwise differences among the means.



Auxin and boron treated existing cells % cell wall thickness analysis

Completely Randomized AOV for C

Source	**DF**	**SS**	**MS**	**F**	**P**
samples	12	799.78	66.6482	1.96	0.0741
Error	26	886.24	34.0860		
Total	38	1686.01			

Grand Mean 23.906 CV 24.42

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	11.0	12	0.5307
Cochran's Q	0.1900		
Largest Var / Smallest Var	26.475		

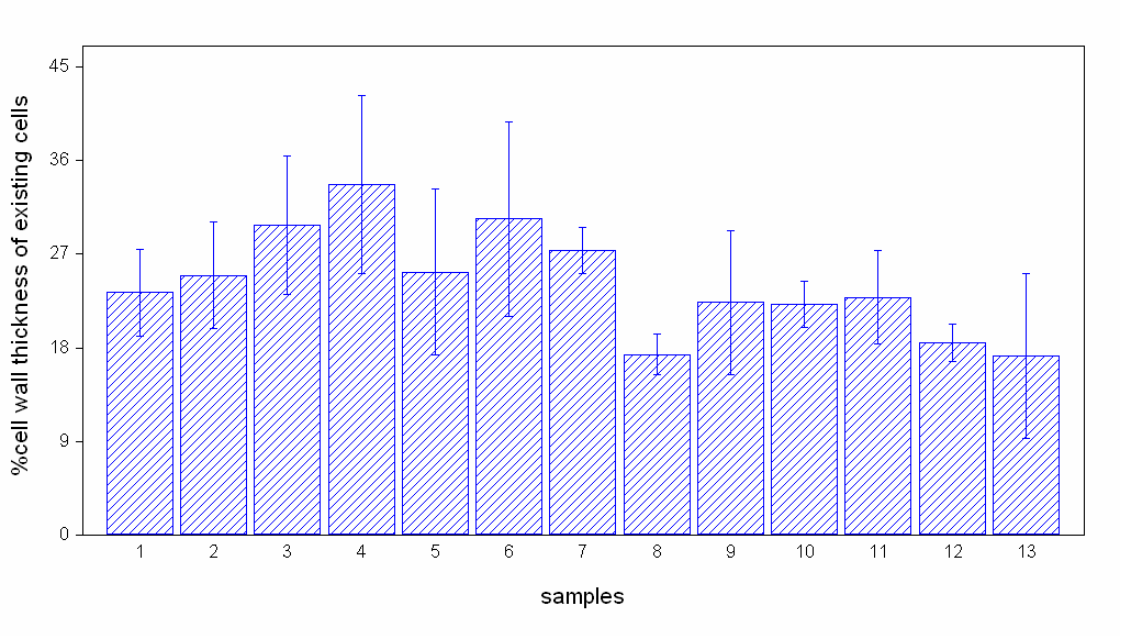
Component of variance for between groups 10.9006
Effective cell size 3.0

samples	**N**	**Mean**	**SE**	**samples**	**N**	**Mean**	**SE**
1	3	23.331	3.3708	8	3	17.331	3.3708
2	3	24.966	3.3708	9	3	22.353	3.3708
3	3	29.792	3.3708	10	3	22.207	3.3708
4	2	33.674	4.1283	11	3	22.868	3.3708
5	5	25.274	2.6110	12	3	18.506	3.3708
6	2	30.414	4.1283	13	3	17.213	3.3708
7	3	27.363	3.3708				

Tukey HSD All-Pairwise Comparisons Test of C by samples

samples	Mean	Homogeneous Groups
4	33.674	A
6	30.414	A
3	29.792	A
7	27.363	A
5	25.274	A
2	24.966	A
1	23.331	A
11	22.868	A
9	22.353	A
10	22.207	A
12	18.506	A
8	17.331	A
13	17.213	A

Alpha 0.05
Critical Q Value 5.138
There are no significant pairwise differences among the means.



Auxin and boron treated %lignin analysis

Completely Randomized AOV for C

Source	DF	SS	MS	F	P
samples	12	52.500	4.37498	1.72	0.0704
Error	122	310.364	2.54396		
Total	134	362.863			

Grand Mean 4.0088 CV 39.79

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	31.4	12	0.0017
Cochran's Q	0.1524		
Largest Var / Smallest Var	19.570		

Component of variance for between groups 0.18080
Effective cell size 10.1

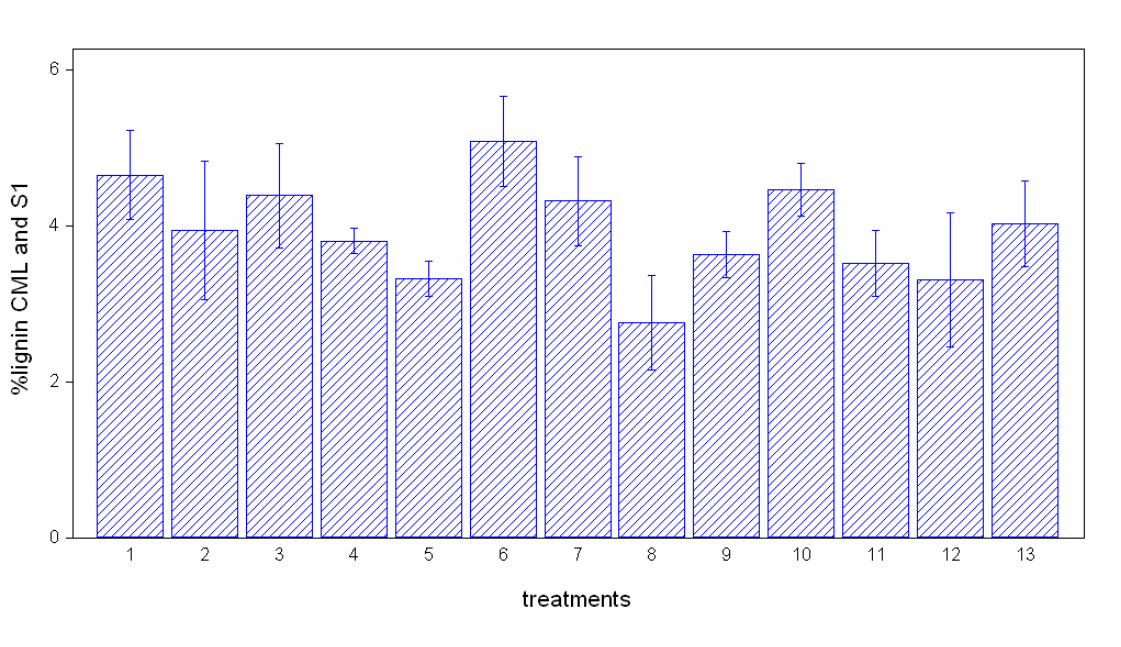
samples	N	Mean	SE	samples	N	Mean	SE
1	15	4.6519	0.4118	8	5	2.7579	0.7133
2	5	3.9460	0.7133	9	15	3.6362	0.4118
3	5	4.3900	0.7133	10	14	4.4682	0.4263
4	10	3.8066	0.5044	11	5	3.5179	0.7133
5	23	3.3232	0.3326	12	5	3.3053	0.7133
6	15	5.0830	0.4118	13	13	4.0302	0.4424
7	5	4.3169	0.7133				

Tukey HSD All-Pairwise Comparisons Test of C by samples

samples	Mean	1	2	3	4	5	6	7
1	4.6519							
2	3.9460	0.7058						
3	4.3900	0.2619	0.4439					
4	3.8066	0.8453	0.1394	0.5834				
5	3.3232	1.3286	0.6228	1.0667	0.4834			
6	5.0830	0.4312	1.1370	0.6931	1.2764	1.7598*		
7	4.3169	0.3350	0.3709	0.0731	0.5103	0.9937	0.7661	
8	2.7579	1.8940	1.1881	1.6321	1.0487	0.5653	2.3251	1.5590
9	3.6362	1.0157	0.3099	0.7538	0.1704	0.3129	1.4469	0.6807
10	4.4682	0.1837	0.5222	0.0783	0.6616	1.1450	0.6148	0.1513
11	3.5179	1.1340	0.4281	0.8721	0.2887	0.1947	1.5651	0.7990
12	3.3053	1.3466	0.6408	1.0847	0.5013	0.0180	1.7778	1.0116
13	4.0302	0.6217	0.0841	0.3598	0.2236	0.7069	1.0529	0.2867

samples	Mean	8	9	10	11	12
8	2.7579					
9	3.6362	0.8783				
10	4.4682	1.7103	0.8320			
11	3.5179	0.7600	0.1183	0.9503		
12	3.3053	0.5474	0.3309	1.1630	0.2126	
13	4.0302	1.2723	0.3940	0.4381	0.5123	0.7249

Alpha 0.05
Critical Q Value 4.679
The homogeneous group format can't be used
because of the pattern of significant differences.



Auxin and boron treated acetyl bromide lignin assay analysis

Completely Randomized AOV for lignin

Source	DF	SS	MS	F	P
CODES	8	95.272	11.9089	0.53	0.8216
Error	17	385.486	22.6756		
Total	25	480.757			

Grand Mean 23.016 CV 20.69

	Chi-Sq	DF	P
Bartlett's Test of Equal Variances	9.07	8	0.3362
Cochran's Q	0.6796		
Largest Var / Smallest Var	240.15		

Component of variance for between groups -3.78289
Effective cell size 2.8

CODES	N	Mean	SE
1	4	21.831	2.3809
2	4	22.157	2.3809
3	4	24.234	2.3809
4	4	23.060	2.3809
5	2	23.489	3.3672
6	2	27.752	3.3672
7	2	24.699	3.3672
8	2	19.758	3.3672
9	2	20.947	3.3672

Tukey HSD All-Pairwise Comparisons Test of lignin by CODES

CODES	Mean	Homogeneous Groups
6	27.752	A
7	24.699	A
3	24.234	A
5	23.489	A
4	23.060	A
2	22.157	A
1	21.831	A
9	20.947	A
8	19.758	A

Alpha 0.05
Critical Q Value 4.999
There are no significant pairwise differences among the means.

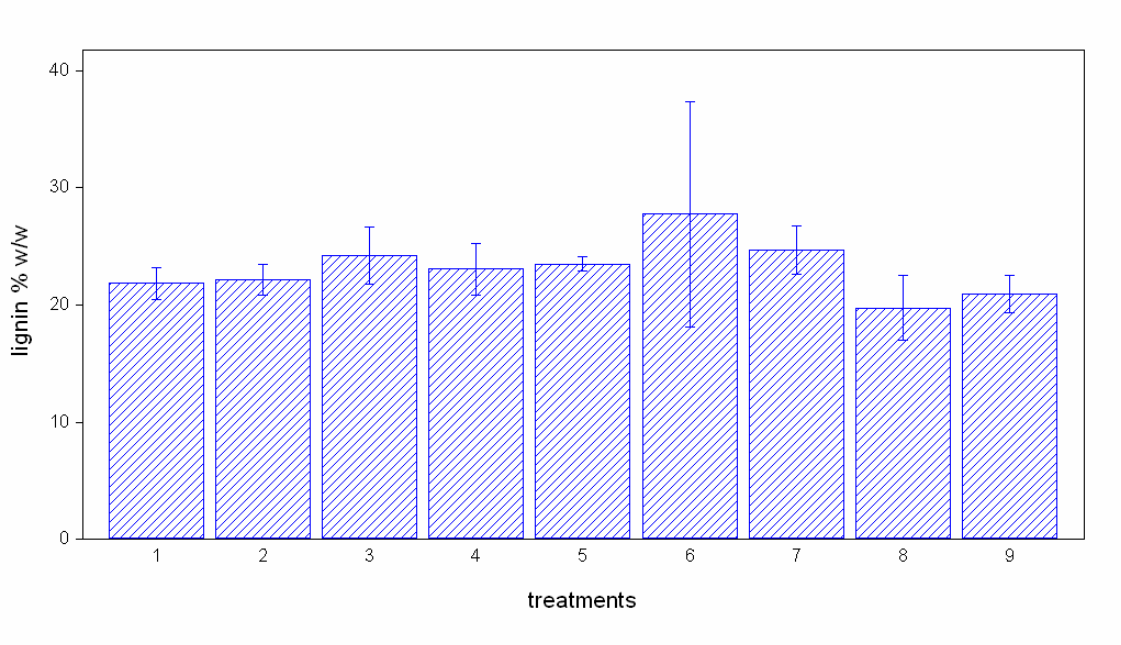


Table 5 *: Composition of lignin and polysaccharides breakdown products identified by the Py-GC-MS. The results are the averages of the duplicate measurements performed on one set of cultures. Values are ± standard error of mean.*

	Culture treatments						
Carbohydrate Products	1µM, 0.03 mM NAA	100 µM, 0.03 mM NAA	1µM, 0.3 mM NAA	100µM, 0.03 mM NAA	1µM, 3 mM NAA	100µM, 3 mM NAA	Control
4-hydroxy-5,6-dihydro-(2H)-pyran-2-one	15.779 ±3.63	15.819 ±4.26	2.35 ±1.22	9.779 ±0.85	3.677 ±1.37	2.625 ±0.91	7 ±0.02
HMF	47.825 ±0.68	43.46 ±0.73	6.01 ±0.89	46.379 ±2.33	6.61 ±0.65	5.337 ±0.21	2 ±0.03
4-allyl phenol	4.263 ±0.25	6.577 ±0.9	0.449 ±0.08	4.204 ±0.07	0.366 ±0.04	0.334 ±0.02	0.02 ±0.04
anhydro gluco pyranose	32.133 ±2.7	34.143 ±2.64	8.8615 ±2.68	39.639 ±1.56	6.836 ±2.84	5.815 ±1.21	9 ±0.5

	Culture treatments						
Lignin Products	1µM, 0.03 mM NAA	100 µM, 0.03 mM NAA	1µM, 0.3 mM NAA	100µM, 0.03 mM NAA	1µM, 3 mM NAA	100µM, 3 mM NAA	Control
guaiacol	22.772 ±0.65	23.409 ±0.88	21.184 ±0.09	22.346 ±0.46	21.965 ±0.59	11.282 ±0.52	19 ±0.01
2-methyl phenol	3.826 ±0.23	4.431 ±0.37	3.362 ±0.17	3.23 ±0.44	2.891 ±0.53	1.7105 ±0.2	2 ±0.4
phenol	12.795 ±0.83	13.214 ±1.02	10.182 ±0.06	10.124 ±1.12	9.754 ±0.81	5.278 ±0.23	7 ±1
4-vinyl guaiacol	25.334 ±0.58	0.265 ±0.13	22.803 ±2.46	25.937 ±0.14	23.32 ±0.8	12.916 ±1.13	18 ±0.1
dihydro coniferyl alcohol	0.797 ±0.02	0.792 ±0.17	2.284 ±0.62	1.437 ±0.24	1.7483 ±0.84	1.289 ±0.28	2 ±0.1
coniferaldehyde	6.385 ±3.41	2.51 ±0.02	7.42 ±1.89	4.256 ±1.07	6.289 ±3.22	4.75 ±1.2223	6 ±0.6
coniferyl alcohol	4.595 ±4.03	0.474 ±0.04	1.88 ±0.95	0.765 ±0.25	1.625 ±1.11	1.37 ±0.51	2 ±0.4
vanillin	41.602 ±29.18	9.822 ±0.49	13.535 ±0.57	37.752 ±26.48	14.534 ±0.93	7.731 ±0.89	8 ±0.5
eugenol	45.083 ±26.41	18.873 ±1.36	17.352 ±1.36	19.968 ±0.347	17.873 ±1.64	9.756 ±0.82	10 ±0.1

Appendix Six

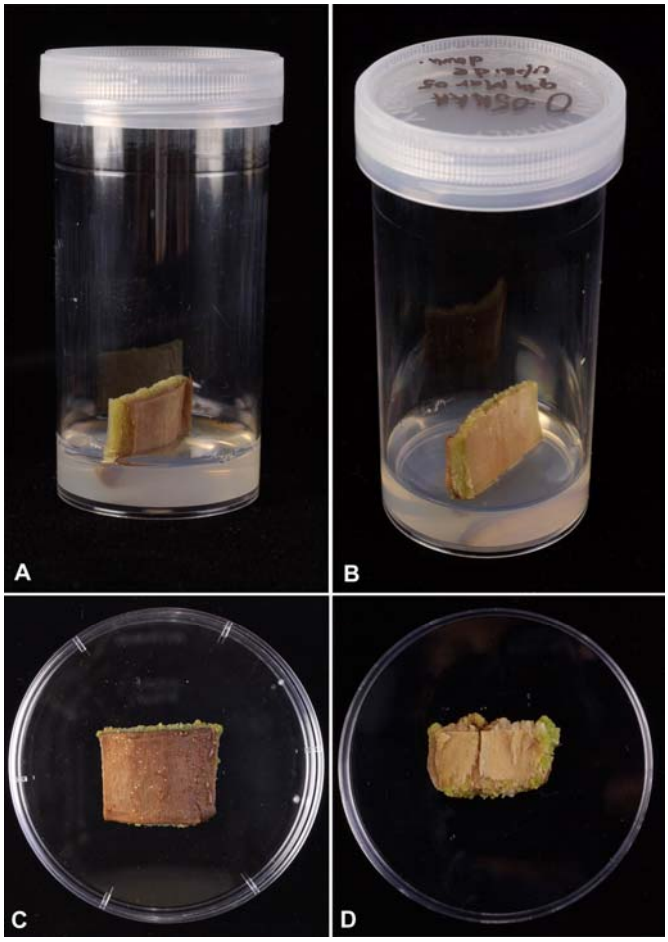
Supplemental auxin culture analysis grown in various orientations

This appendix presents the details of the data presented in of the organ cultures that were grown in various concentrations to understand the role of auxin transportation.

Set No. 1 Upright	0.05 NAA	0.5 NAA	5 NAA	50 NAA	control	0.05 NAA	0.5 NAA	5 NAA	50 NAA
culture date 5th Mar 05									
Growth Period: 1 month									
Changes in cell number									
Cambial region	4 ±0.63	5.8 ±2.78	4.25 ±0.5	5.5 ±0.84	2.2 ±0.4	3.8 ±0.84	4.2 ±1.11	3.8 0.45	4.5 ±0.58
RE region	14 ±2.97	10.4 ±1.95	7 ±0.82	9.83 ±0.41	7.6 ±1.52	11.2 ±4.32	11.8 ±2.17	15.4 ±1.14	14.5 ±2.65
New secondary wall region	8 ±1.79	7.6 ±0.55	10.5 ±0.58	7.67 ±0.82	6.8 ±1.3	8.8 ±1.1	5.4 ±1.14	8.2 ±1.3	6.25 ±0.96
Cambial region cells									
lumen area (μm^2)	218.65 ±72.52	201.56 ±77.05	174.17 ±97.83	211.57 ±92.51	269.26 ±84.23	280.96 ±58.19	171.71 ±61.59	161.42 ±52.71	162.55 ±69.33
radial length (μm)	7.73 ±1.25	8.07 ±1.68	11.45 ±1.98	7.98 ±2.09	8.79 ±2.168	9.21 ±1.46	7.51 ±1.86	7.02 ±1.63	6.68 ±1.59
tangential length (μm)	27.88 ±6.24	24.63 ±6.97	23.16 ±7.51	26.014 ±6.93	30.28 ±4.27	30.58 ±4.33	22.66 ±5.53	22.85 ±4.58	23.59 ±5.88
RE region cells									
lumen area (μm^2)	568.33 ±157.3	518.72 ±206.8	578.23 ±218	695.58 ±229	555.33 ±249.9	525.24 ±170.9	680.3 ±208.5	640.21 ±243.9	693.69 ±278.4
radial length (μm)	32.67 ±5.39	30.31 ±6.065	31.85 ±6.27	35.96 ±6.74	31.34 ±6.88	33.21 ±7.07	34.13 ±4.74	32.05 ±5.88	33.95 ±6.53
tangential length (μm)	22.73 ±4.04	21.64 ±4.76	22.54 ±5.64	25.37 ±5.4	22.45 ±5.96	21.21 ±5.55	26.15 ±5.56	25.2 ±6.32	25.91 ±6.48
cell wall area(%μm^2)	62.47	45.78	35.99	39.13	35.47	26.91	23.09	24.78	21.99

Existing cells									
lumen area (μm^2)	679.04 ±350.7	743.9 ±327.8	481.19 ±415.9	629.46 ±522.2	778.32 ±388.4	620.55 ±333.1	729.85 ±353.9	669.66 ±289.1	944.38 ±459.6
radial length (μm)	36.43 ±8.04	35.47 ±6.96	28.94 ±13.26	33.47 ±16.23	40.057 ±7.8	35.79 ±9.98	34.89 ±7.88	33.49 ±6.89	40.054 ±10.39
tangential length (μm)	23.8 ±8.08	25.89 ±6.72	18.49 ±12.16	21.92 ±12.17	26.37 ±9.01	22.47 ±7.33	25.57 ±7.66	24.74 ±7.65	29.31 ±9.38
cell wall area(%μm^2)	-	24.75	54.49	42.95	43.56	53.1	15.03	29.56	34.21
lignin area in CML/$S_{1(}$(% lignin μm^2)	4.32			8.1	3.79	8.25			4.2
Set No. 2 culture date 27th Mar 05 Growth Period: 1 month	0.05 NAA	0.5 NAA	5 NAA	50 NAA	control	0.05 NAA	0.5 NAA	5 NAA	50 NAA
Changes in cell number									
Cambial region	2.4 ±0.55	4 ±0.63	4 ±1.09	4 ±0.9	2 ±0	4.4 ±0.89	4 ±1.26	4.2 ±0.84	5 ±0.71
RE region	10.6 ±1.2	11.67 ±1.75	13.17 ±1.83	17 ±2.76	10.67 ±0.82	10.6 ±2.7	12.83 ±2.56	13.2 ±4.97	9.6 ±0.9
New secondary wall region	5.4 ±0.49	6.83 ±1.33	8.33 ±1.03	5.5 ±0.55	6.33 ±0.52	7.4 ±1.52	6.67 ±1.03	5.6 ±1.14	7 ±0.71
Cambial region cells									
lumen area (μm^2)	189.22 ±73.15	170.52 ±74.03	181.34 ±95.87	206.77 ±68.02	210.09 ±74.24	215.78 ±65.73	228.17 ±105.1	190.43 ±94.82	192.12 ±59.26
radial length (μm)	6.84 ±1.86	6.19 ±1.59	6.28 ±2.13	6.88 ±1.72	7.18 ±1.96	6.65 ±2.39	7.4 ±2.42	7.58 ±2.03	6.49 ±1.5
tangential length (μm)	27.69 ±7.089	26.93 ±7.30	28.27 ±8.49	30.53 ±7.64	28.97 ±4.055	33.59 ±6.29	29.93 ±6.66	24.41 ±7.89	29.5 ±5.39
RE region cells									
lumen area (μm^2)	518.42 ±174.5	484.39 ±154.2	531.99 ±192.9	542.42 ±176.9	595.14 ±182.9	628.22 ±212.7	664.67 ±229.9	393.31 ±154.4	530.71 ±197.2
radial length (μm)	31.43 ±5.82	31.55 ±5.67	32.99 ±6.49	30.98 ±5.47	31.33 ±5.068	32.55 ±6.7	33.31 ±5.88	28.97 ±6.12	30.08 ±5.37
tangential length (μm)	20.27 ±4.03	20.26 ±3.62	21.46 ±4.62	21.28 ±3.87	23.86 ±4.90	23.03 ±3.48	24.39 ±5.38	18.5 ±4.23	22.42 ±4.99
cell wall area(%μm^2)	37.77	49.52	52.55	42.58	39.28	39.55	42.73	63.04	44.16

Existing cells									
lumen area (μm^2)	847.34 ±369.4	684.44 ±396.4	799.79 ±334.4	569.07 ±272.9	785.08 ±359.4	650.95 ±309.4	770.37 ±330.5	664.78 ±314.6	1009.1 ±331.3
radial length (μm)	38.88 ±8.58	36.91 ±10.4	39.66 ±7.87	34.05 ±7.46	35.52 ±7.77	32.57 ±7.36	37.69 ±8.29	35.01 ±8.19	45.54 ±7.56
tangential length (μm)	26.55 ±6.92	22.6 ±8.28	25.43 ±7.28	22.64 ±6.88	26.73 ±7.33	24.36 ±7.33	25.08 ±7.29	24.01 ±6.86	27.83 ±5.68
cell wall area(%μm^2)	28.43	35.28	34.19	23.29	33.9	30.22	28.29	26.49	29.69
lignin area in CML/S_1(%lignin μm^2)	4.77			14.74	4.66	5.45			10.07



Experimental design

The organ cultures were grown vertically with their apical end upwards and their basal ends downwards. In this way the basipetal flow of auxin was maintained as in an intact tree (A). The direction of the transport of auxin was disrupted by growing cultures upside down so that the basal end was upwards and the apical end downwards touching the media (B). The organ cultures were grown horizontally with either the existing wood (C) or phloem (D) in contact with the media.

APPENDIX SEVEN

Supplemental data of the comparative analysis with respect to mechanical properties of the checked and non-checked wood

The following appendix contains the supplemental data that was reported to the Wood Quality Initiative Limited, New Zealand. It summarises the data of the investigations that were carried out to study some of the mechanical properties of wood.

EXECUTIVE SUMMARY

DENSITY, MICROBRIL ANGLE AND MOE AS INDICATORS OF INTRA-RING CHECKING.

Hema Nair and Sandra Jackson

Report No. App 1.6.2, 1.6.3 and 1.6.21 **Date: August 2005**

The problem

Intra-ring checking is the result of the collapse of fibres in the radial dimension. Earlier studies have pointed out that the wood that checked varied in the cellular dimensions, ultrastructure and cell wall chemical composition compared to the non-checked wood. All these features in turn can exert influence on the mechanical properties of wood and in turn the susceptibility of wood to checking.

WQI Initiative

The goal of this study was to compare the mechanical properties of checked and non-checked wood in an attempt to see if the checked wood differed in mechanical properties that made it more susceptible to checking. This study will be able to throw some light on the influence of mechanical properties of wood on checking.

This Project, (Report No. APP 1.6.2, 1.6.3 and 1.6.21)

The mechanical properties like density, microfibril angle (MFA) and modulus of elasticity (MOE) of checked and non-check wood were examined and data collected. The observations for the density, MFA and MOE were collected by SilviScan-2. MFA was also examined with the help of X- ray diffraction and MOE data was also obtained with the help of modified Fullams micro- test stage. The observations and analysis of the density MFA and MOE could help us ascertain the role of the mechanical properties of wood and their influence on checking.

Results

The checked wood had lower density as compared to the non-checked wood as per the SilviScan-2 data. We saw similar trends with the measured density data analysis, even though the values obtained from the two data varied. However, density is considered not a very good property to be used as predictive tool. Previous research has pointed out that higher density measurements could be a

result of compression wood or false growth ring that can be present in the wood sample. The MFA was higher for checked wood when measured as per X- ray diffraction method. The SilviScan-2 data analysis did not show clear differences between the non-checked wood and checked wood. Hence we cannot conclude without doubt that high MFA can lead to checking. The MOE showed no trend as per the Siliscan-2 data between checked and non-checked wood. However, as per modified Fullams micro- test stage observation we could see that checked wood had lower MOE as compared to non-checked wood, but the relationship was not statistically significant. Overall on closer analysis of the data no clear picture emerges of differences between checked and non-checked wood.

From the data analysis of density, MFA and MOE we can see that there is not one clear property is more influential than the other in causing checking.

Implications for WQI

From the study it becomes evident that checking in wood is complex and could be result of combination of whole lot of factors coming together. The results show that there is not one single mechanical property alone that could clearly be the reason for the wood to develop checking on drying. This report is an indicator that there is a need for taking a more holistic approach to understanding and solving the intra ring checking. It is more reliable to use the mechanical properties in combination with the cellular ultrastructural properties and cell wall chemical composition to predict about checking in wood.

INTRODUCTION

Wood finds its many uses in our day-to-day life, however, when wood is being used for construction, furniture and in other industrial applications then other synthetic products challenge its mechanical properties. A better understanding of the mechanical properties will help us in better utilisation of wood. Some of the previous work indicated that the wood that checked wood was different from the non-checked wood with respect to the cellular dimensions, ultrastructure and cell wall chemical composition (Jackson and Nair, 2003, WQI Report App 10, Jackson *et al*., 2004, WQI App 34). This study is directed towards exploring how the mechanical properties of checked wood are different from non-checked wood and if there is any kind of weakness that exists in checked wood making it more susceptible to checking.

Experimental Design

The study was carried out on the thirteen oven-dried discs of radiata pine displaying a range of intra-ring internal checking and no checking (see WQI App 10, Fig 1). WQI App 10 and WQI App 34 reported the anatomical and histochemical analyses of these discs. In the current comparative study the mechanical properties of the wood discs with severe checking(3 discs), moderate checking(6 discs) and wood that displayed no checking(4 discs) in the growth rings were compared.

Pieces of wood were cut out of oven dried discs of radiata pine and were observed, with help of SilviScan-2, X- ray diffraction and modified Fullams micro- test stage. Samples were prepared from same growth ring and used to measure density and MOE and MFA. In order to minimise the variations between the growth rings we limited our study to growth ring 7(details of the methods are given in Appendix 1).

Objectives

- Main objective was to determine if the checked wood had different mechanical properties as compared to non-checked wood.

- Determine the relationship between mechanical properties and its role in checking.

RESULTS AND DISCUSSION

It is likely that low density wood is more susceptible to checking

Density of wood can influence developments of internal checks (Simpson *et al.*, 2002; Illic, 1999 a; b; Chafe, 1994). If density is low, there is increase in checking (Simpson et al., 2002; Illic, 1999). The checked wood seemed to have lower density compared to the non-checked wood from the analysis of the SilviScan-2 and the calculated density data. There is a possibility that lower the density of wood the more susceptible the wood will be to checking. There are differences in the values of the data obtained from these methods. However, the trends between the checked wood density and the non-checked wood are almost similar (see Table 1). Overall, internal checking shows negative relationship to density. The lower the density of wood the more likely the wood will develop checks (SilviScan-2 ANOVA P= 0.0003 and the measured density ANNOVA P= 0.06, see Appendix 2). The observations from sample 1 were not taken into consideration for analysis due the presence of the false growth ring that was observed in the ring 7 of the sample disc 1 that could affect the measurements of the property.

Density is one of the important properties of wood, but is certainly not that simple. It is quality trait that is function of xylem anatomy and hence affected by cell wall thickness, cell diameter, and the chemical content of wood (Zobel& Van Buijtenen, 1989; Cave&Walker, 1994; Lundgren, 2004). Hence it seems that the cell wall thickness and composition could play important role in influencing density of wood and in turn checking. As per Simpson *et al.*(2002) higher the wood density thicker the cell wall lower the tendency of the wood to check. Thick cell walls ensure that only small compressive stresses occur in the cell walls. Such cells can resist collapse more efficiently and prevent development of internal checks. We have already discussed this in more detail in our previous reports (Jackson and Nair, 2003, WQI Report App 10; Jackson *et al*., 2004, WQI Report,

App 34) where we saw that checked wood had thinner cell walls and hence, more prone to collapse of cells and checking in wood.

The high density wood is most likely to have cell walls that are thick and in addition have small cell lumens. When the water tension acts on the free water in cell lumen, the combination of small lumen diameter and thick strong cell walls ensures that only small compression stresses occur in the cell wall and hence the cell walls do not collapse and prevent checks from developing in wood (Simpson *et al*., 2002). As previously reported (Jackson and Nair, 2003, WQI Report App 10) the cell lumens in the checked wood were larger as compared to non-checked wood. The cell lumen can influence water movement during drying and in turn affect development of checks in wood (Jackson *et al*., 2004, WQI Report, App 34). The latewood tissue usually does not check and one of the possible reasons could be that the latewood tissue have cells with relatively small radial diameter, thick wall, small cell lumen and, therefore have higher density than thin walled earlywood cells with larger cell lumen (Haygreen& Bowyer, 1996). However, earlywood density plays a major role in growth ring density as the proportion of earlywood is more in growth ring compared to latewood (Donaldson et al., 1995), and hence its cellular dimensions need careful attention.

Calculated density			Density as per SilviScan-2		
check	Average density	sample size	check	Average density	sample size
severe	**424.57** ±112.15	30	severe	**369.9** ±30.9	490
moderate	**360.7** ±93.98	119	moderate	**356.77** ±36.38	1369
no check	**433.54** ±89.22	77	no check	**396.7** ±25.87	860

TABLE 1: *Density data analysis of severe, moderate and non-checked wood as per SilviScan-2 and calculated density (kg/m^3) at 40% RH and 20°C. The values obtained from the two methods are different. However, the trends between the checked and non-checked wood are same.* ***The checked wood showed lower density values as compared to the non-checked wood.***

Interrelationship between collapse, shrinkage, density and internal checking

Shrinkage and collapse are negatively related to density (Illic, 1999 a; b; Chafe, 1985, 1986). Collapse of the wood fibres can lead to internal checking (Simpson *et al.*, 2002, Illic, 1999 a; b; Chafe, 1994). Earlier studies have reported (Jackson *et al*., 2004, WQI Report, App 34, Singh and Donaldson, 2000) that the samples with checked wood displayed presence of collapsed fibres along the edge of the check and away from the check (see Fig 6). While observing the SilviScan-2 samples under the dissecting microscope we found collapsed cells in checked wood. Whereas in the non-checked wood we saw very few collapsed cells. From

the density data and the microscopy data of the same samples it becomes evident that lower density wood is more inclined to collapse and shrink as compared to high density wood. In material susceptible to collapse (as per Chafe, 1985 and 1986) probably like the checked wood, the thickness of the cell wall is critical in resisting liquid-tension forces associated with drying. Cells with thin walls, low density, collapse and shrink more.

We do have the results that point in the direction that checked wood does display low density that can translate to thin cell wall and large lumen. However, in spite of these results caution must be exercised while making predictions about whether a particular wood is more prone to checking or not, based on density of wood alone. High density displayed by a wood could also possibly be due to the presence of compression wood or false growth ring.

Higher MFA in wood could increase the possibility of checks developing in the wood.

Cave and Walker(1994) stated that MFA is major determinant of stiffness in wood as compared to density. Higher MFA result in low stiffness and greater longitudinal shrinkage and both these properties are important for checking

check	Average MFA (degrees)	sample size
severe	**41.2** ±2.59	5
moderate	**34** ±2	5
non check	**27.8** ±4.49	5

(Butterfield, 1998 and Butterfield& Pal, 1998).

The X-ray diffraction data shows that the checked wood had higher MFA as compared to the non-checked wood (Table 2). The values and the trends obtained from the SilviScan-2 data are different from the X- Ray diffraction data. The MFA values for the checked wood as per SilviScan-2 data are lower than the non-checked wood (Appendix 9, Table 8). One of possible explanation for this kind of discrepancy in the data could be the presence of the collapsed cells that could have influenced the data obtained from the SilviScan-2 measurements that were seen during microscopy
on the same samples and the presence of checks in the samples.

Table 2: *MFA data analysis obtained from X-Ray diffraction.* ***As per X-Ray diffraction the MFA of checked wood was higher than the non-checked wood.***

The X- Ray diffraction is also able to measure the crystalline nature of cellulose present in the cell wall. On analysis of the data obtained for X-Ray diffraction we see that the cellulose of the checked wood had lower crystallinity as

compared to the non-checked wood (Table 3). The levels of cellulose crystalline nature showed a gradual increase from the severe to the non-checked wood. The intensity of the cellulose was lowest in severe wood compared to moderate and non-checked wood (Table 3). From the data recorded, we could also see that the checked wood had more noise in the data compared to the non-checked wood. The non-checked wood displayed almost smooth cellulose peaks and less noise (represented by big black arrows and arrowheads in Fig 1). The X-Ray diffraction data indicates that the cell wall organisation with regards to cellulose organisation seems to be more uniform and cohesive in the non-checked wood compared to the checked wood.

check	Average crystallinity of cellulose	Cellulose intensity	Sample size
severe	**0.25** ±0.028	**96250** ±19384.14	5
moderate	**0.36** ±0.04	**153060** ±33168.71	5
non check	**0.42** ±0.05	**126980** ±12553.76	5

TABLE 3: Analysis of data obtained from *X- ray diffraction on cellulose crystallinity and intensity in checked and non-checked earlywood.* ***The cellulose had lower level of crystallinity and intensity in checked wood as compared to non-checked wood.***

MFA can also influence the fracturing properties of wood. Small MFAs favour transwall fracture as opposed to intrawall fracturing under transverse shear (Donaldson, 1998). In our pervious study we did see that the check usually occurred along the compound middle lamella and S_1 cell wall layer. The predominant form of fracturing that we observed was intrawall fracturing (Jackson *et al*., 2004, WQI Report, App 34) at the site of check. Hence it seems that the high microfibril that we see in the checked wood as per X- ray diffraction data makes the wood more vulnerable to fracture between the walls and lead to checking.

Theoretical analysis reveals that at low angles the lignin-hemicellulose matrix has no influence on the axial stiffness. On the other hand when MFA exceeds about 40° the matrix alone determines the axial stiffness and this remains constant for larger MFA (Walker& Woollons, 1998). At large angles the axial stiffness is low and is controlled by the shear properties of the matrix of hemicelluloses and lignin (Huang *et al*., 2003). The checked wood has high MFA 41.2°. Therefore, in this case the lignin-hemicellulose matrix could influence development of checks in wood. From our previous study we know that checked wood showed lower levels of lignin (Jackson *et al*., 2004, WQI Report, App 34,

Jackson and Nair, 2003, WQI Report App 10). It could be possible that the high MFA and low lignin content in the wood could make the wood more susceptible to checking.

Relationship between MFA, shrinkage and checking in wood.

The MFA also exerts its control on other wood properties like stiffness and shrinkage. The stiffness of cell wall increases as the MFA decreases (Cave& Walker, 1994; Walker& Butterfield, 1995; Evan&Illic, 2001; Downes *et al*., 2002; Deresse *et al.*, 2003; Barnett&Bonahm, 2004; Lindstrom *et al*., 2002 and 2004). The longitudinal shrinkage increases with MFA but in non linear manner (Walker&Butterfield, 1995). Researchers have shown that MFA has been linked to dimensional changes in wood with changes in moisture content (Preston, 1942; Harris& Meylan, 1965; Meylan, 1968). As the wood undergoes drying the water that is bound to the cellulose and hemicellulose moves out of the cell wall. These molecules come closer and the wood shrinks (Barnett&Bonham, 2004). If the MFA is small, then most shrinkage will take place transversely and as there is increase in the MFA there will be an increase in longitudinal shrinkage (Huang *et al*., 2003; Barnett&Bonham, 2004, Lundgren, 2004).

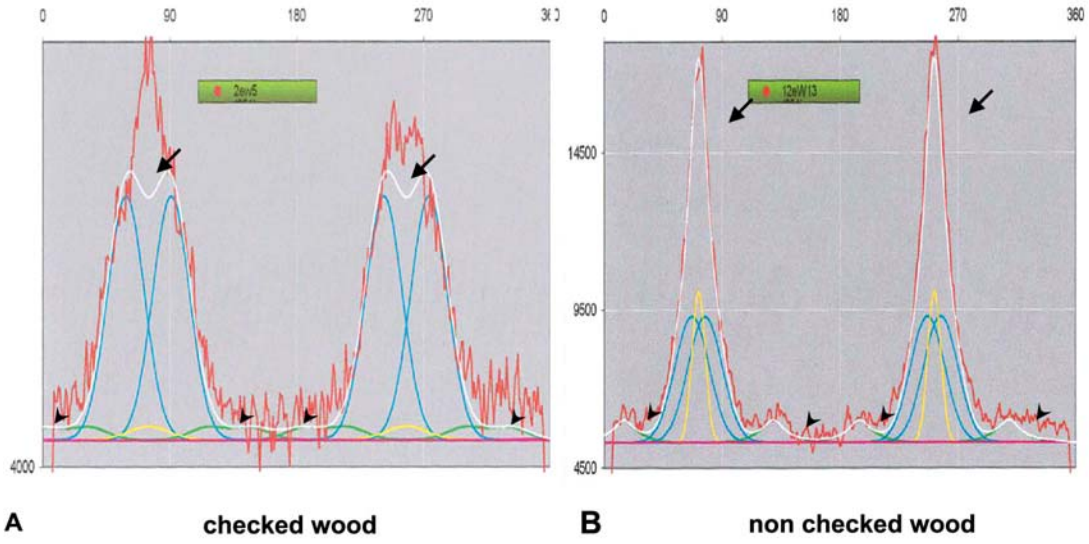


Fig 1: *The figure shows that X- Ray diffraction pattern of a checked wood. The black arrows and arrowheads in the figure represent the cellulose intensity and cellulose organisation in the wood.* ***The non-checked wood (A) shows smooth peaks, more cohesive and uniform cellulose organisation as compared to the checked wood (B).***

The larger MFA means that the tracheids will shrink excessively in the longitudinal direction during drying compared to normal wood (Huang *et al*.,

2003). The cellulose microfibrils restrain the cell wall from shrinking in the direction parallel to their axis during drying and are pulled together laterally whereas the non- crystalline matrix of hemicellulose and lignin shrink more extensively in the transverse plane leading to anisotropic shrinkage. These changes in wood during drying could lead to differential shrinkage that can lead to stresses in the wood which could cause development of wood defects including checking (Huang *et al*., 2003; Barnett&Bonham, 2004). The minimum longitudinal shrinkage in radiata pine occurs at 25° (Wang et al., 2001). As per X-ray diffraction data the non-checked wood showed an average MFA of 27.8°compared to the checked wood average MFA of 34° and 41.2°. Therefore it seems likely that the checked wood on drying would have a tendency to undergo greater differential shrinkage causing stresses in wood that might lead to checks in the wood. However, just like density we cannot use MFA alone to predict shrinkage in wood. We have been warned about against using MFA measurement alone to estimate longitudinal shrinkage in wood, as factors other than MFA can also influence shrinkage like degree of lignification and the extent to which internal growth stresses have accumulated in the tree and are released when a tree is cut (Megraw et al., 1998 and Barnett&Bonham, 2004).

Overall it seems that lower the MOE the more susceptible the wood will be to checking.

MOE is one of the most important mechanical properties of solid timber applications. MOE is largely influenced by MFA (Cave & Walker, 1994; Walker& Butterfield, 1995; Hirakawa *et al*., 1998; Evans & Ilic, 2001; Yang & Evans, 2003) and density (Panshin & de Zeeuw, 1980; Cown *et al*., 1999). MOE is a composite function of MFA and density (Evans & Ilic, 2001; Lindstrom *et al*., 2002& 2004). Density is positively correlated to MOE (Cown, et al., 1999) and it accounted for 81 percent of variation in MOE (Yang & Evans, 2003). However, density alone cannot explain the variations of MOE in trees (Yamashita *et al.,* 2000). MFA has high correlation to MOE as compared to density (Cave & Walker, 1994; Evans & Ilic, 2001; Linstrom *et al*., 2002&2004). MFA accounted for 87 percent of variation in MOE (Yang & Evans, 2003). MFA and density jointly accounted for 92 percent of MOE variations (Evans & Ilic, 2001; Yang & Evans, 2003). Both static and dynamic MOE were found to be primarily dependent on MFA (Linstrom *et al.*, 2004; 2002).

Checked and non-checked wood MOE was observed using data from SilviScan-2 and modified Fullams micro-test stage. In case of the data from the SilviScan-2 there were no clear trends between the checked wood and the non-checked wood (Fig 2). However, the data from the modified Fullams micro-test stage showed a vague sort of trend, where, the checked wood had lower MOE compared to the non-checked wood (Fig 3). Though the data was not statistically significant (Fullams result for ANOVA P=0.18 and the SilviScan-2 ANOVA P= 0.32, see Appendix 2).

One of the reasons for such variable result could be that the wood samples that were prepared behaved independently, depending on their respective cell wall mechanical properties. The behaviour of each sample was further affected by differences in the cell dimensions of the cells in the samples. When SilviScan-2

wood samples were observed with the help of dissectoscope we found that, there were cells that were collapsed, and some that were partially collapsed. In addition to these some differences in the lignification of the cells in the wood could have affected the values of the data obtained. Another factor that could affect the data could be the presence of the checks in some of the samples. The point of failures while doing the fullams micro-test experiment could also be localised and the readings affected. The wood fails at the weakest tracheid layer in the early wood cells with thinnest cell wall (Muller *et al.*, 2003) and could be one of the reasons for such variations in the data obtained. While preparing samples for the Fullams stage the samples are cut into smaller sticks and this could lead to broken tracheids being exposed along the edges of the samples being used for the measurements. There is also possibility of the contamination of data from these broken tracheids that could lead to the variations in the observations.

The mechanical behaviour of the cell walls is also influenced by more ductile lignin- hemicellulose matrix (Bergander & Salmen 2000). Gindl *et al.,* (2002) suggested that as the wood cells undergo secondary wall development the cellulose- hemicellulose primary structure of the cell walls get filled with lignin and the density of the cell wall is believed to increase along with an increase in the MOE. Though lignin is less stiff than the composite polysaccharide structure it may be considered equally as hard. Hence lignin content may contribute to the MOE of wood. We know from our earlier report that the checked wood had lower levels of lignin as compared to non-checked wood. It seems that the severely checked wood could have low MOE as it has higher MFA, low density and also lower levels of lignin in the cell walls. Higher MFA and low density resulted in lower strength of wood (Lundgren, 2004).

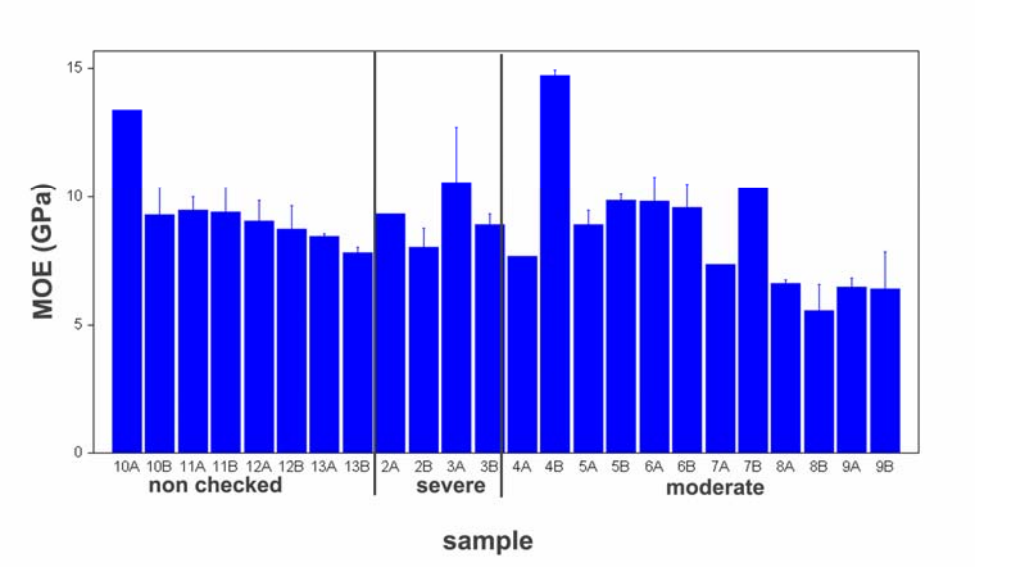


Fig 2: *SilviScan-2 MOE data analysis of the checked and non-checked wood. Sample 10A, a non checking wood and sample 4b, a moderately checked wood showed the highest MOE values.* ***Overall the non-checked wood samples show MOE very close to each other compared to the checked samples that show variability in the MOE values from high to low in both the moderate and severely checked wood category.***

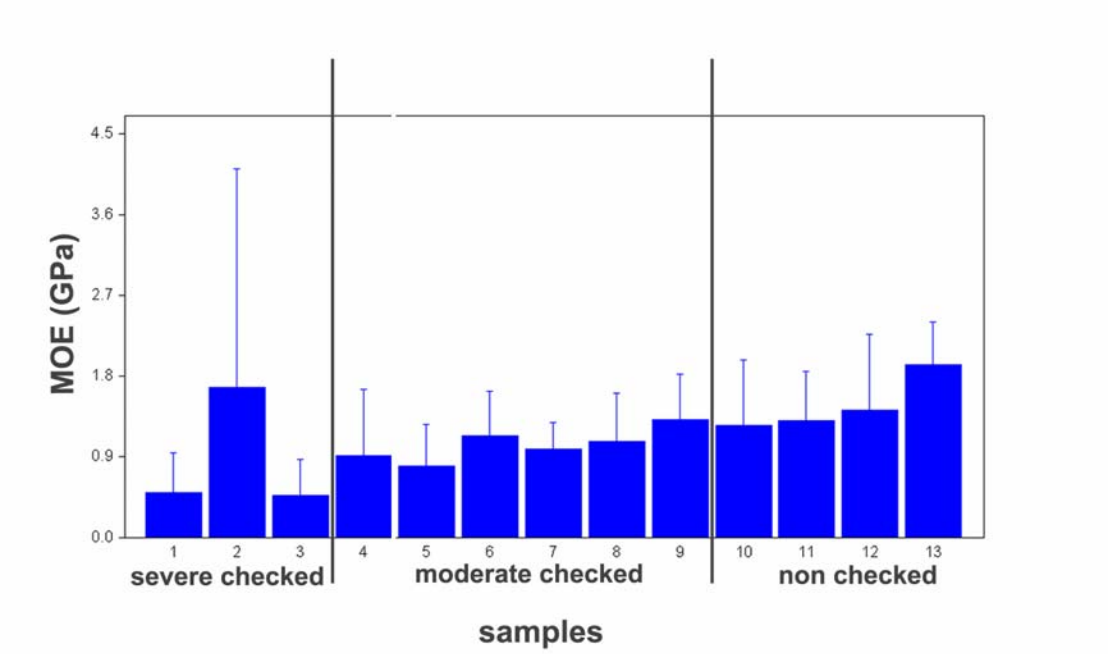


Fig 3: *The modified Fullams micro-test stage MOE data analysis showed results that were similar to the SilviScan results.* ***The non-checked samples had MOE values quite similar to one another compared to the checked wood samples that had high to low MOE values.***

The lower the strength of wood cells the more readily they will collapse under the drying stresses that develop in wood that could lead to formation of checks in the wood.

SHRINKAGE, COLLAPSE AND CHECKING

Internal checking was significantly related to collapse (Ilic, 1999b, Singh& Donaldson, 2000) and shrinkage (Innes, 1996a). A high positive correlation between number of checks and shrinkage gives and indication of the predisposition by the material towards shrinkage as a whole (Chafe, 1994). Collapse alone explained 47 percent of the variation in internal checking (Ilic, 1999). Internal checking showed significant positive relationship with collapse and total shrinkage in *E regnans* (Ilic & Hillis, 1986; Ilic 1999). In low density wood it has been suggested that collapse severity and internal checking tends to be greater in growth rings with thin earlywood cell walls (Ilic, 1999). Both total shrinkage and collapse are negatively related to basic density and positively related to moisture content, and moisture content in turn is negatively related to basic density (Ilic, 1999). Hence a low density wood will have high moisture content, higher shrinkage and collapse that could lead to checks in wood.

Typically when internal checks occur, they are spaced at short tangential distances. High differential levels of tensile stress must be able to develop locally to initiate such checks. This development is consistent with collapse occurring in low strength (low density) earlywood bands in the presence of relatively high restraint from denser latewood (Ilic, 1999a). From the data analysis earlier we did see that the severely checked wood showed low density, high MFA and low MOE.

It seems likely that the cells are unable to withstand the stresses that develop during drying, and lead to development of checks.

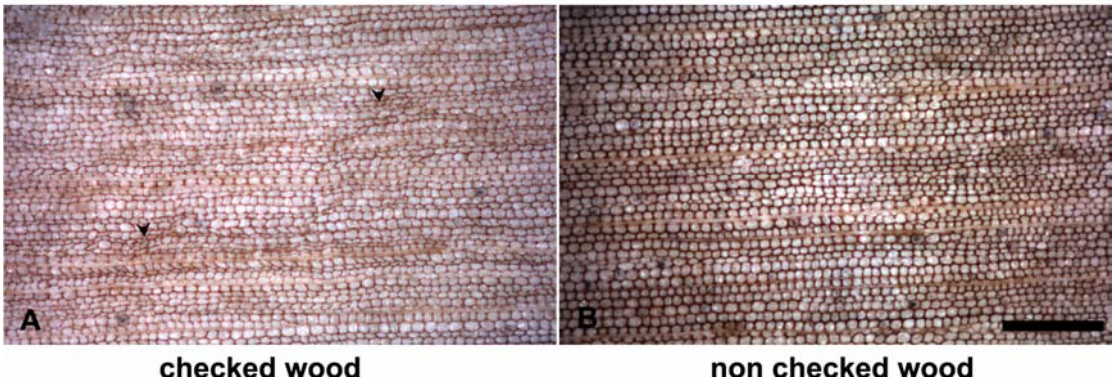


Fig 4: *Light microscopy of the SilviScan-2 samples.* ***The micrographs revealed that the checked wood displayed collapsed cells (pointed out by black arrowheads in A), while the non-checked wood did not show collapsed cells (B).***

Collapse occurs during drying as water is removed from wood cells (Simpson *et al.*, 2002, Illic, 1999 a). During drying as water is removed from highly impermeable wood fibres they become distorted because of high tensile forces generated in the lumen water and in some case cave in and collapse (Ilic, 1999a). There were a number of collapsed cells that were seen in the checked wood samples when we did the microscopy observations on the SilviScan-2 specimens. Similar observations of collapsed cells associated with checked wood were made by Singh and Donaldson (2000). So it is likely the checks that we see in our samples of the radiata pine oven dried discs could have developed in a similar manner.

The severely checked wood (Fig 5A) displays curling of the edge inwards due to high differential shrinkage compared to the non-checked wood (Fig 5C) that has an almost straight edge and hence has undergone minimal differential shrinkage while drying. The moderate wood shows an intermediate stage (Fig5B) where the edges curl inwards but not to the same extent as the severely checked discs. These observations indicate that there is high likelihood of the checked wood to have undergone more differential shrinkage during drying.

High MFA results in low stiffness and increased longitudinal shrinkage. Both these properties are particularly relevant for fast grown species (Butterfield, 1998; Butterfield & Pal, 1998) like radiata pine. A large longitudinal shrinkage value means greater change in length with change in moisture content, which can itself be detrimental. Harris& Meylan (1965) showed that longitudinal shrinkage increases while tangential shrinkage decreases at MFA greater than 25°. MFA tends to decline in the last few latewood tracheids but values in the earlywood of the next growth ring are much higher creating a very steep gradient across the boundary. The large difference in the MFA between cells on either side of ring boundary may mean that differential shrinkage during drying will generate significant shear along boundary which could contribute to ring shakes and other checking phenomenon. This effect may also be influenced by differences in basic density and cell dimensions between adjacent latewood cells and earlywood cells.

The checked wood in our study had very high MFA and low density hence the cells were subjected to high differential shrinkage stresses as they dried and this could have lead to the formation of checks in the wood.

In reality tangential shrinkage in radiata pine, generally exceeds radial shrinkage. This derives from presence of ray cells, from the effect of pit apertures in the radial walls and possibly from difference in microfibril orientation in radial and tangential walls (Astley, 1998). The checked wood had higher ray tissue and larger pit apertures compared to non-checked wood (Jackson *et al*., 2004, WQI Report, App 34). There is a possibility that the rays and pit could have influenced shrinkage and play a role in the formation of check. Similar positive correlation between ray volume and transverse shrinkage was seen in *Cryptomeria japonica* (Kazita *et al*., 1953). At the same time Boutelje (1962) was of the opinion that the rays had no influence on shrinkage anisotropy in conifers but were important in broad rayed timber like oaks. The shrinkage and elastic anisotropy in the conifers can also be accounted for by the distribution and the nature of the cell wall substances (Boutelje, 1962), the proportion of the thick walled fibres in the tissue, the thickness of S2 relative to S1, and variations in lignification of the cell walls layers in the wood (Boyd, 1977).

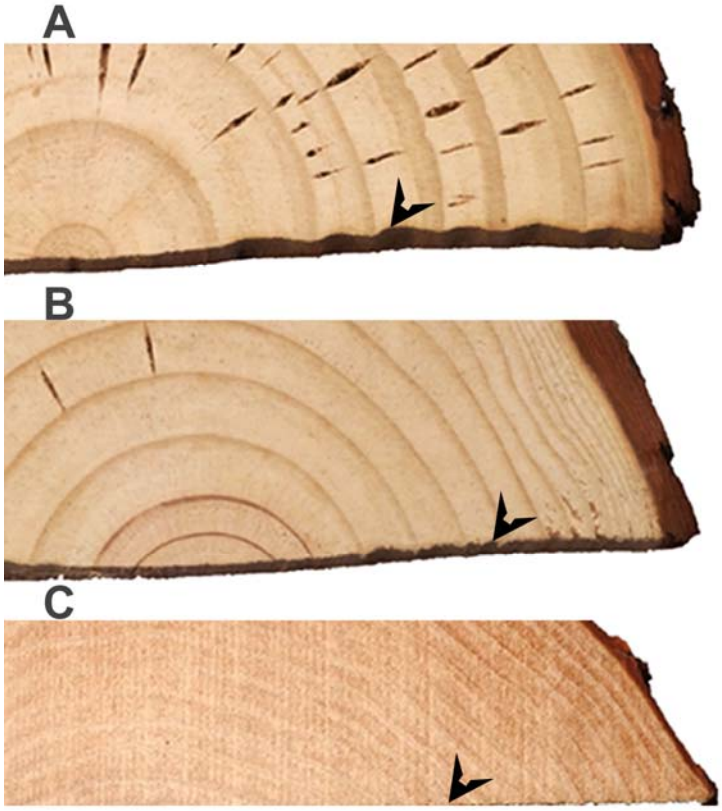


Fig 5: ***The wood that checks seems to undergo more differential shrinkage compared to normal wood****. As is evident from the figure the severely checked (disc A) wood seemed to have undergone more shrinkage on drying and this observation can be made from the inward curling of the disc edges (pointed by*

the arrow in the figure). The moderately checked (disc B) wood has shrunk to lesser extent and the non-checked (disc C) wood the least

Checks are known to form in wood due to high levels of differential shrinkage in local regions, and it is likely that they will be initiated in stress concentration zones of poorly lignified vessels and ray cell wall contact (Mackay, 1972; Ilic, 1998(in Ilic 1999a and Ilic 1999b). If the lignin content is low in the cell walls the compression strength of the wood including the capability of the cell walls to resist collapse during drying is lowered and could lead to collapse of cell (Singh & Donaldson , 2000). One of the possible explanations for this is that when wood is heated to high temperatures the lignin in the wood is softened above its glass transition and there are changes in the chemical structure of lignin. These changes in lignin and its lower content in the cell wall can make the wood more susceptible to checking (Singh & Donaldson, 2000).

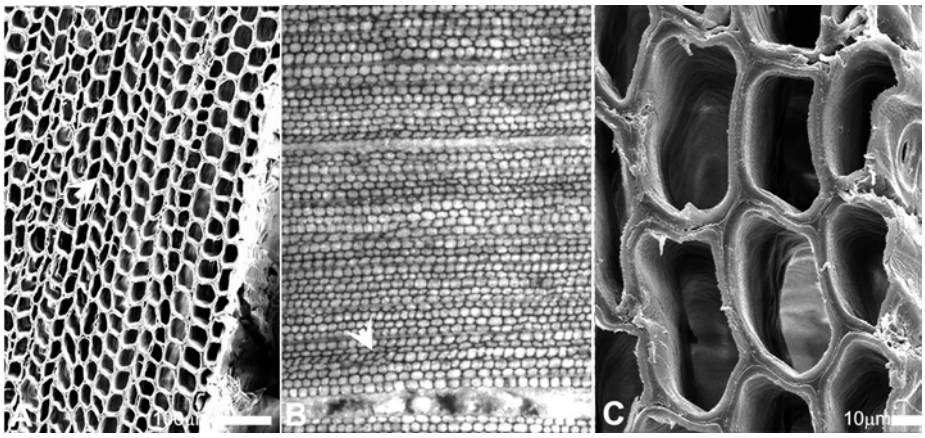


Fig 6*: Collapsed cells were observed along the check and away from the check.* ***A is a scanning electron micrograph of cells seen in checked wood. We can see some cell files that have collapsed and are away from the check (the arrow points to one such cell file). B in the figure displays the collapsed cells in the SilviScan-2 sample as seen under a dissectoscope where collapsed cells could also be seen away from the check (the arrow is used to help point to collapsed cell file). C in the figure is scanning electron micrograph of the collapsed cells observed along the check.***

As mentioned earlier (see section on MFA earlier in this report) when MFA exceeds about 40° the matrix alone determines the axial stiffness (Walker& Woollons, 1998). The checked wood as per the X-ray data analysis has high MFA. Hence during the process of drying when the wood is subjected to the differential shrinkage stresses it will be the lignin hemicellulose matrix that will be important for the cell walls to resist the negative pressures that will develop within cell at the time of drying. The checked wood had lower levels of lignin in the cell wall making them more vulnerable to the stresses leading to the formation of checks. In a comparative study between the hardwood and the softwood it was found that the shrinkage in hardwood was more than in softwood. One of reason for the shrinkage was attributed to the low lignin content of hardwood (Schroeder, 1972).

Lignin seems to be play an important role in cells resistance to collapse and shrinkage, however other anatomical features, like tracheid length, shape and diameter change rapidly with distanced from pith towards bark and their effect on wood properties need to be clarified (Saranpaa *et al.*, 1998).

SWELLING OF WOOD

Swelling of wood could be the possible reversibility of dimensional changes that took place in wood during shrinkage. It has been seen that the dimensional changes that took in wood during drying recovered during swelling (Lawrence, 2004). In one of the drying experiments conducted (Ilic, 1999) it was found large surface checks developed during initial stages of drying and these checks closed tightly after stress reversal towards end of drying. We wanted to see what would happen to the checked wood samples that we had in case of swelling.

We cut out intra-ring checks from the disc of severe and moderately checked wood and measured the width of the checks when dry and when they were almost 100% saturated with water. We did the same to bigger pieces of wood with few growth rings across to see if how the checks are going to be influenced in normal growth ring conditions where they are bounded by latewood bands on either side.

Severely checked wood did not show as much recovery as compared to moderate wood on swelling.

The widths of the check in the severely checked dried wood were wider then the non- checked wood. When the checks were measured after almost 100 percent saturation with water we found that the width of the checks became smaller and in some moderately checked wood closed almost completely. We found that the severe checks on swelling did not recover as much as compared to the moderate checks. In 13.33% cases of the moderately checked wood the checks recovered fully and closed up totally. Similar results were observed with the checks in the discs. We think that the closing of the checks is the possible shrinkage recovery of the cells that happens during swelling. In case of the severely checked wood we did see collapsed cells along with shrinkage. The collapsed cells in our opinion do not recover form their dimensional changes during swelling but the cells that show shrinkage are able to recover some their dimensional changes during swelling and hence we the closure of the check.

Cell walls are saturated with water even in dead cells of freshly felled timber the water content of the wall is normally about 30% on a dry weight basis. The cell wall shrinks as the water is removed and swells on rewetting. The swelling will not be the same as shrinkage. There is going to be some hysteresis, if a wall is kept dry for a long time, and then there is possibility that it may not recover its original dimensions on rewetting. This may be due to the non availability of hydrogen bonds that have been satisfied internally and are no longer available to water. The crystal lattice of cellulose I is not penetrable and not available to water so that swelling and shrinkage per se are confined to the matrix (Preston, 1974).

It is suggested that lignin might be a factor that influences shrinkage recovery (Bland, 1971). When wood undergoes drying various deformations occur and the wood properties will be modified. The heat treatments can result in softening of hemicellulose and lignin, and cross-linking of components maybe a

result of this softening. To some extent these deformations can be reversed by soaking. A theory based on different cross-linking mechanisms between the components in the wood may explain the reversal of dimensions (Sandland, 1999). Unusually thick walled fibres were associated with visco-elastic strain recovery effects, which could form a substantial part of dimensional changes apparently attributable to shrinkage (Boyd, 1977). Necesany (1966) was of the opinion that lignin did play a role in the shrinkage of cell wall and that middle lamella was also an important factor influencing both the swelling and shrinkage of cells. Lignin in the cell walls could have a tendency towards restraining effect against dimensional change (Schroeder, 1972). The lumen of the earlywood tracheids expanded, whereas the lumen of latewood tracheids shrank, indicating a greater influence of the secondary wall over restraining middle lamella in latewood (Quirk, 1978). The checked wood had lower lignin and thinner cell walls as compared to the non-checked wood it seems that these two features of the cell wall can influence the not only the mechanical properties of wood but also have am important role to play in the shrinkage and swelling of wood and cellular deformations.

check	sample no	Average % closure of check width	Sample size
severe	1	**45.93** ±12.44	35
severe	2	**46.17** ±22.57	23
severe	3	**71.11** ±11.43	8
moderate	4	**70.68** ±12.9	29
moderate	5	**76** ±28.41349	7
moderate	6	**82.31** ±18.7	9
moderate	7	**58.8** ±11.56	6

Table 4*: The data shown in the table below is percentage average of the proportion of the check width closure in the checked wood samples. The check width was measured when the wood was dry and measured again when the wood was 100% saturated with water.* ***The width of the checks in the moderately checked wood recovered or became smaller as compared to the severely checked wood.***

The whole wood shrinks and swells at high MFA (Preston, 1974). One of possible reason for this was explained on the basis of distribution of water mainly between the concentric lamellae of microfibrils. Burmester (1972) also indicated that anisotropy of swelling increases with increasing moisture content. As the matrix of the dry wood swells it puts the non swelling microfibrils under tension and itself under compression (Barber and Meylan, 1964). When the MFA in the cell

wall are small the microfibril are in the form of flatter helices and they will shrink and swell more longitudinally. However, when MFA is high they are in the form of steeper helices in the cell wall and on either shrinkage or swelling will cause more tension and there is more movement in wood (Preston, 1974). These movements in wood could possibly lead to wood defects like checking.

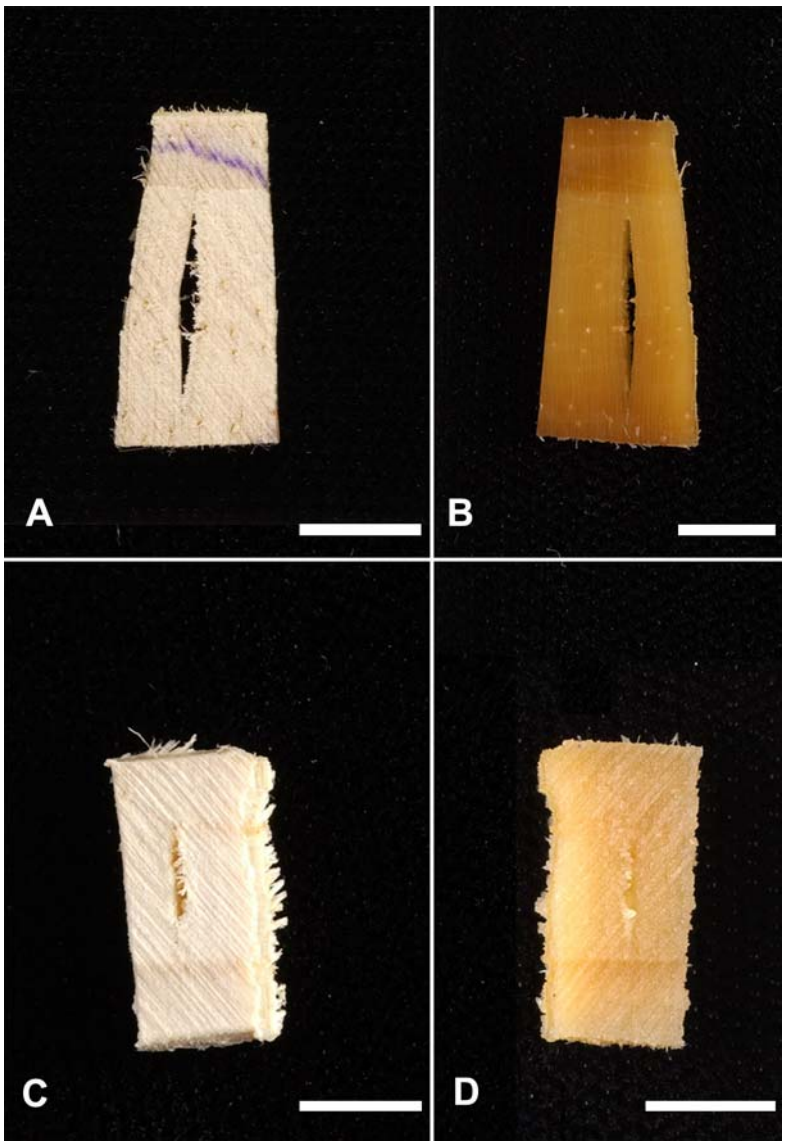


Fig 7: *The digital photographs of the checks width when dry and when 100%saturated with water.* ***The width of the checks in the moderately checked wood recovered or became smaller(C and D) as compared to the severely checked wood (A and B).***

CONCLUSIONS

We found that there were no clear differences that were evident between the checked and non-checked wood depending on their mechanical properties. As is

evident from the data analysis there were trends with some data and none with others. The SilviScan-2 data as far as the density of wood was concerned did show differences and a statistically significant relationship, the checked wood had lower density as compared to non-checked wood. However, the same relationship did not hold true when density was recorded by the second method, though we did see the trend of checked wood having lower density compared to non-checked wood. Similarly for MFA the SilviScan-2 data did give much insight into the differences between checked and non checked wood, however X- ray diffraction showed that checked wood had higher MFA than non-checked wood. Overall the data obtained from X-ray diffraction had higher MFA values than the SilviScan-2 data. The data analysis for checked wood for MOE obtained from SilviScan-2 and modified Fullams micro- test stage did not show any statistically significant relationship between MOE and checking. However we did see sort of a trend in the Fullams data where the checked wood had lower MOE compared to non-checked wood.

There seems to be a possibility that the hemicellulose and lignin matrix might also have an affect on the mechanical properties of wood and could influence checking. It also became evident that the properties like density and MOE were influenced by cellular dimensions like thickness of the cell wall and cell lumen. The cell wall properties of wood are mainly influenced by the mechanical properties of fibre and matrix material.

RECOMMENDATIONS

Lignin-hemicellulose lignin matrix might affect the mechanical properties of wood and influence checking.

Lignin seems to be exerting its influence on not only the mechanical properties of wood but also on some other very important properties of wood like shrinkage, swelling, and cellular deformations of wood that are of importance from the point of view of development of checks in wood.

- Further analysis on the changes in that take place in lignin when wood is heated to high temperatures would be able to throw more light on the role of lignin and its affect on properties of wood during drying process. A special attention could be given to the lignin in the wood above its glass transition temperature as there are changes in the chemical structure of lignin.
- Lignin plays a role in the shrinkage of cell wall, and shrinkage can influence checking, more work in this direction can help us to understand what the relationship between the two is, and in what is their role in checking of wood.

Prediction of quality of wood and closer look at the methods

We tried to examine the same property of wood using different methods. From the study we know that we get different values for the same property of wood. One has to be careful about making judgments on the quality of wood based on the data obtained from one method alone. It seems like a combination of tools and taking more than one property of wood into consideration might help in making more accurate predictions about the quality of wood.

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APPENDIX 1

Materials and methods

Plant material

Thirteen oven dried discs were kindly provided to WQI Ltd by Graeme Young.
The discs were grouped into severe, moderate and no check samples based on the number of visible checks.

The specimens were then prepared analysis on these instrument SilviScan-2, X- ray diffraction and Fullam's stage. For modified Fullams micro- test stage and X- Ray diffraction the samples were obtained from growth ring 7 to minimise variations in growth ring.

SilviScan sample collection:

Wood pieces were cut from the original discs so as to comprise of as many growth rings as possible and sent for SilviScan measurements on the mechanical properties of wood like MOE, MFA and density.

SilviScan measurements(Evans, 1998):

The pieces of wood are cut into radial strips of dimensions 2mm tangentially by 7mm longitudinally. These are the dimensions used by SilviScan- 2 for image analysis and microdensiometry. All the measurements are made in conditioned atmosphere maintained at 40%RH and 20°C. The samples are conditioned from zero moisture content, having been dried with ethanol and then extracted with acetone. Samples are examined with the x- ray beam in the tangential direction. The growth ring orientation is measured by automated image analysis and the information can be used by the control software to maintain the growth rings parallel to the beam. The sample or the growth rings may be held at nominated fixed angle to the x-ray beam.

Image analysis and physical strength requirements dictate a sample thickness (tangential direction) between 1mm and 2mm. The absorption contrast for microdensiometry is done with copper Kα on average for wood samples approximately 2mm thick. The analysis rate depends on the chosen spatial resolution, which is currently limited to the 0.2mm diameter for the x-ray beam. Radial profiles with 0.2mm step size are obtained at the rate of 30mm/hour. To avoid problems associated with variation in fibre axis orientation, the integration span is usually limited to 10mm, on the assumption that fibre orientation is constant within the chosen span.

A copper rotating anode in point focus mode is used in conjunction with a nickel filter and a capillary focussing system to produce a beam cross-section of diameter *ca* 0.2mm at the sample. The diffraction patterns are recorded with a CCD area detector. The strong (002) equatorial reflection is used for MFA analysis. Useful images of the equatorial reflections can be obtained in as little as

10seconds, although 30seconds is commonly used to improve the signal to noise ratio.

Modified Fullams micro- test stage sample preparation:(Butterfield and Pal, 1998):

Wood pieces were cut from original discs with the help of chisel and hammer. The top and bottom transverse cut surfaces of wood were then smoothened using sledge microtome to form a wood block of 4mm (MSE microtome). These blocks were then cut further into 1mm wide strips, and then cut again for 1mm at right angles to these slices using a specially designed engineers press with rotating stage (Olympus, Japan model no. 219722). All the sticks are then examined under a microscope and those with any cutting defects rejected. A minimum of 20 sticks per sample were selected for testing.

The sticks were first weighed and measured for length, height and width at 40% humidity. The sticks were then compression load tested in modified Fullams micro-test stage in a 40% humidity environment. The load cell and the linear transducer (for measuring linear compression) are interfaced to personal computer fitted with data acquisition board and running locally written software. Maximum crushing strength and stiffness are automatically recorded by the PC. The figures for the stiffness were taken from central part of the load/displacement graph so as to avoid any distortion caused by end collapse of cut cells during initial compression loading.

APPENDIX 2

ANOVA Tables
Nested ANOVA's were carried out using S Plus statistical package.

SilviScan-2 analysis of density (kg/ m^3) of severe, moderate and non-checked earlywood samples measurements obtained at 40% RH at 20° C

Error: as.factor(sample.no)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
checking	2	845323.4	422661.7	12.21	0.0003035337
Residuals	21	726936.3	34616.0		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	2698	2128946	789.083		

Tukey HSD All-Pairwise Comparisons Test of DENSITY for check

check	**Mean**	**Homogeneous Groups**
3	476.33	A
1	365.16	B
2	347.74	B

Alpha 0.05 Standard Error for Comparison varies
Critical Q Value 3.314 Critical Value for Comparison varies
Error term used: Error, 224 DF
There are 2 groups (A and B) in which the means
are not significantly different from one another.

Analysis of density (kg/ m^3) measurements of severe ,moderate and non-checked earlywood taken at 40% RH at 20° C

Error: as.factor(sample)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
check	2	403784.0	201892.0	3.896639	0.06039232
Residuals	9	466306.4	51811.8		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	217	3077727	14183.08		

SilviScan-2- 2 analysis of density (kg/ m^3) of severe, moderate and non-checked latewood samples measurements obtained at 40% RH at 20° C

Error: as.factor(sample.no)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
check	2	87224 4361	2.09	0.5337083	0.5941716
Residuals	21	1716020	81715.22		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	1311	15757872	12019.73		

Analysis of density (kg/ m^3) measurements of severe ,moderate and non-checked latewood taken at 40% RH at 20° C

Error: as.factor(sample)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
check	2	662602.7	331301.4	6.18417	0.02042315
Residuals	9	482152.4	53572.5		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	200	3437526	17187.63		

Tukey HSD All-Pairwise Comparisons Test of DENSITY for check

check	**Mean**	**Homogeneous Groups**
3	644.63	A
1	555.77	B
2	520.98	B

Alpha 0.05 Standard Error for Comparison varies
Critical Q Value 3.314 Critical Value for Comparison varies
Error term used: Error, 209 DF
There are 2 groups (A and B) in which the means
are not significantly different from one another

SilviScan-2 analysis of difference between latewood and earlywood density (kg/ m^3) measurements of severe ,moderate and non-checked wood taken at 40% RH at 20° C

Error: as.factor(sample.no)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
checking	2	446667	223333.3	1.427698	0.2893803
Residuals	9	1407861	156429.0		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	1277	13866457	10858.62		

Analysis of difference between latewood and earlywood density (kg/ m^3) measurements of severe ,moderate and non-checked earlywood taken at 40% RH at 20° C

Error: as.factor(sample)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
check	2	93193.0	46596.51	0.6993026	0.5220395
Residuals	9	599695.5	66632.84		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	113	1705321	15091.34		

SilviScan-2- 2 analysis of MOE (GPa) of severe, moderate and non-checked wood earlywood samples.

Error: as.factor(sample.no)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
checking	2	837.766	418.8831	1.17189	0.3292345
Residuals	21	7506.291	357.4424		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	2698	2110.036	0.782074		

Analysis of the MOE (GPa) data of severe,moderate and checked wood earlywood samples obtained from modified Fullams micro-test stage.

Error: as.factor(sample)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
check	2	8.582630e-016	4.291314e-016	2.058864	0.1835387
Residuals	9	1.875881e-015	2.084312e-016		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	217	1.796199e-014	8.277413e-017		

SilviScan-2- 2 analysis of MOE (GPa) of severe, moderate and non-checked wood latewood samples

Error: as.factor(sample.no)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
check	2	180.478	90.2389	0.2495777	0.7814081
Residuals	21	7592.896	361.5665		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	1311	466.416	0.3557711		

Analysis of the MOE (GPa) data of severe,moderate and checked latewood samples obtained from modified Fullams micro-test stage.

Error: as.factor(sample)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
check	2	1.393381e-015	6.966903e-016	2.314457	0.154531
Residuals	9	2.709150e-015	3.010167e-016		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	200	1.921574e-014	9.607869e-017		

SilviScan-2- 2 analysis of difference between latewood and earlywood MOE (GPa) of severe, moderate and non-checked wood samples.

Error: as.factor(sample.no)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
checking	2	216.214	108.1069	0.7364979	0.5055593
Residuals	9	1321.066	146.7851		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	1277	4137.578	3.240077		

Analysis of the difference between latewood and earlywood MOE (GPa) of severe, moderate and non-checked wood samples obtained from modified Fullams micro-test stage.

Error: as.factor(sample)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
check	2	4.751467e-016	2.375733e-016	2.674431	0.1225781
Residuals	9	7.994823e-016	8.883140e-017		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)

Residuals	113	1.034153e-014	9.151795e-017		

Analysis of SilviScan-2- 2 readings of MFA(degrees) of severe, moderate and non checked wood samples

Error: as.factor(sample.no)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
checking	2	305.15	152.573	0.08898267	0.9152045
Residuals	21	36007.39	1714.638		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	2698	2505.489	0.9286469		

SilviScan-2- 2 analysis of difference between latewood and earlywood readings of MFA(degrees) of severe, moderate and non-checked wood samples.

Error: as.factor(sample.no)

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
checking	2	141.085	70.5426	0.1980174	0.8238355
Residuals	9	3206.200	356.2444		

Error: Within

	Df	Sum of Sq	Mean Sq	F Value	Pr(F)
Residuals	1277	8648.169	6.772254		

APPENDIX 3

The weighted averages of the earlywood data obtained from the SilviScan-2.

Sample	Radius (mm) length	Conditioned density (20°C;40% RH (~7%MC) (kg/m3)	radial diameter (um)	Tangential diameter (um)	Coarseness (ug/m)	Wall thickness (um)	Microfibril Angle (deg.)	MOE (GPa)
01A	67.2	**477.3** ±110.0	**36.5** ±4.1	**29.4** ±1.9	**504.6** ±100.8	**2.8** ±0.6	**21.0** ±3.5	**9.6** ±2.8
01B	82.9	**450.3** ±119.6	**36.2** ±3.1	**28.1** ±1.7	**453.5** ±107.4	**2.6** ±0.7	**23.2** ±3.9	**7.9** ±2.8
02A	92.9	**478.4** ±137.9	**36.4** ±5.1	**29.5** ±1.9	**499.9** ±105.9	**2.8** ±0.8	**18.9** ±5.6	**11.3** ±4.5
02B	105.1	**438.5** ±106.7	**35.7** ±3.6	**28.3** ±1.8	**436.8** ±88.6	**2.5** ±0.6	**26.3** ±3.4	**6.7** ±2.2
03A	103.8	**387.7** ±108.0	**37.6** ±3.9	**30.9** ±1.4	**441.3** ±88.3	**2.3** ±0.6	**14.5** ±1.8	**9.5** ±2.5
03B	86.7	**436.8** ±105.0	**36.6** ±4.1	**32.0** ±1.7	**500.4** ±80.6	**2.7** ±0.6	**17.4** ±2.3	**9.8** ±1.2
04A	68.7	**456.6** ±122.5	**37.7** ±5.1	**31.8** ±1.9	**529.7** ±75.0	**2.8** ±0.6	**12.8** ±1.9	**12.8** ±3.0
04B	97.9	**484.0** ±134.0	**35.1** ±4.5	**29.1** ±1.9	**483.0** ±98.6	**2.8** ±0.7	**12.0** ±1.1	**14.1** ±2.7
05A	103.5	**430.9** ±102.9	**38.9** ±3.7	**31.0** ±1.6	**511.1** ±89.2	**2.7** ±0.6	**21.1** ±1.9	**8.6** ±1.8
05B	85.5	**488.9** ±113.3	**39.2** ±4.2	**31.6** ±1.9	**592.5** ±91.2	**3.1** ±0.7	**18.9** ±2.4	**10.8** ±1.5
06A	101.7	**424.5** ±103.7	**38.5** ±3.9	**29.8** ±1.4	**476.5** ±75.9	**2.5** ±0.6	**18.2** ±2.0	**9.5** ±1.8
06B	104.8	**447.0** ±107.4	**38.0** ±4.2	**30.3** ±1.6	**505.7** ±88.3	**2.7** ±0.6	**18.9** ±3.7	**9.7** ±2.4
07A	58.9	**441.6** ±118.5	**35.2** ±4.7	**31.4** ±2.0	**471.5** ±63.7	**2.6** ±0.6	**11.6** ±0.8	**12.5** ±2.5
07B	56.0	**445.0** ±118.1	**35.5** ±4.8	**31.5** ±2.1	**480.8** ±63.1	**2.6** ±0.6	**11.3** ±0.6	**13.3** ±1.5
08A	89.7	**430.9** ±128.4	**34.6** ±3.5	**27.1** ±1.7	**394.3** ±79.2	**2.3** ±0.7	**21.7** ±5.3	**7.3** ±3.1
08B	97.9	**427.0** ±131.1	**35.1** ±3.7	**27.5** ±1.6	**402.2** ±89.7	**2.4** ±0.7	**20.7** ±4.6	**7.3** ±3.1
09A	125.1	**371.6** ±101.2	**38.4** ±3.5	**32.8** ±1.8	**461.5** ±103.1	**2.3** ±0.6	**19.6** ±4.0	**8.2** ±2.4
09B	104.2	**364.1** ±102.7	**38.1** ±3.5	**32.2** ±1.7	**440.1** ±101.3	**2.3** ±0.6	**19.6** ±2.6	**7.7** ±2.1
10A	86.1	**511.9** ±153.4	**36.9** ±5.1	**30.1** ±2.0	**547.0** ±88.4	**3.1** ±0.8	**13.2** ±1.4	**14.0** ±2.3

10B	100.1	**506.4** ±169.7	**36.5** ±5.0	**29.8** ±1.6	**526.6** ±88.1	**3.0** ±0.9	**12.7** ±1.4	**13.9** ±2.3
11A	100.0	**462.1** ±107.5	**35.7** ±4.3	**31.4** ±2.2	**504.5** ±70.7	**2.8** ±0.5	**16.7** ±1.9	**10.7** ±2.5
11B	99.5	**455.2** ±99.6	**35.2** ±4.3	**30.9** ±2.2	**486.3** ±77.8	**2.7** ±0.5	**16.2** ±1.7	**10.8** ±2.2
12A	100.8	**466.0** ±140.7	**34.7** ±4.2	**29.3** ±1.8	**462.2** ±96.6	**2.7** ±0.7	**16.9** ±2.9	**10.6** ±2.1
12B	99.5	**469.5** ±140.0	**34.0** ±4.2	**28.3** ±1.9	**440.7** ±94.0	**2.6** ±0.7	**15.7** ±2.5	**10.8** ±2.3
13A	88.8	**445.5** ±88.6	**35.3** ±3.4	**29.3** ±1.6	**456.8** ±79.0	**2.6** ±0.5	**23.6** ±3.3	**7.9** ±1.9
13B	88.8	**459.0** ±92.5	**35.5** ±3.7	**29.5** ±2.0	**476.1** ±90.1	**2.7** ±0.5	**24.4** ±3.3	**7.7** ±2.0

APPENDIX 4

The SilviScan-2 earlywood averages of density, MFA and MOE of individual samples growth ring 7.

Sample number	Check type	Avg density kg/ m3	Avg MFA (degrees)	Avg MOE (GPa)	sample size
1A	severe	**428.5959** ±6.886683	**17.92** ±7.34E-15	**14.607** 0	15
1B	severe	**274.877** ±66.90264	**20.1** ±3.74E-15	**12.5615** ±1.87E-15	10
2A	severe	**344.3235** ±24.49887	**20.52** ±1.07E-14	**9.32473** ±1.79E-15	63
2B	severe	**379.3707** ±38.32045	**23.45841** ±0.737522	**8.01638** ±0.742799	131
3A	severe	**356.4389** ±32.12241	**13.41537** ±1.038228	**10.57938** ±2.133444	146
3B	severe	**383.4517** ±25.60232	**17.88203** ±1.414768	**8.906046** ±0.441964	147
4A	moderate	**371.0621** ±25.23869	**13.12** ±1.79E-15	**7.6904** ±3.57E-15	82
4B	moderate	**367.8818** ±20.13511	**11.61615** ±0.509387	**14.71507** ±0.199337	51
5A	moderate	**391.8386** ±27.18604	**19.95647** ±0.161117	**8.899098** ±0.578516	115
5B	moderate	**391.286** ±27.30136	**16.38417** ±0.263127	**9.860897** ±0.230304	71
6A	moderate	**359.0242** ±24.7236	**16.21176** ±1.170818	**9.811977** ±0.979145	187
6B	moderate	**365.7095** ±23.52987	**15.47875** ±0.621358	**9.582562** ±0.921296	119
7A	moderate	**362.2527** ±52.11278	**12.24** ±7.15E-15	**7.3644** ±5.37E-15	73
7B	moderate	**363.857** ±34.82986	**12.02** ±8.97E-15	**10.3629** ±8.97E-15	52
8A	moderate	**358.2296** ±18.349	**21.36565** ±0.125018	**6.63682** ±0.116738	123
8B	moderate	**355.3149** ±21.1778	**21.79559** ±1.479506	**5.552472** ±1.031018	117
9A	moderate	**328.0299** ±33.18302	**23.24948** ±2.186272	**6.494788** ±0.332896	171
9B	moderate	**326.665** ±44.49032	**22.12023** ±1.833261	**6.387389** ±1.507228	197

10A	non check	**385.9467** ±22.00365	**13.24** ±7.17E-15	**13.3726** ±3.58E-15	56
10B	non check	**401.4657** ±9.654147	**16.57084** ±1.066328	**9.30069** ±1.059882	94
11A	non check	**399.4941** ±21.15956	**18.30727** ±0.527214	**9.45999** ±0.531094	98
11B	non check	**389.1439** ±21.75815	**18.12652** ±0.480279	**9.384385** ±0.952565	137
12A	non check	**398.7412** ±28.31687	**18.02807** ±0.983065	**9.056619** ±0.807755	134
12B	non check	**406.0229** ±40.85363	**19.82309** ±0.364113	**8.737678** ±0.893813	138
13A	non check	**399.3775** ±17.15087	**22.25539** ±0.121791	**8.460675** ±0.10794	101
13B	non check	**387.1499** ±15.75926	**24.77792** ±0.059399	**7.802118** ±0.237464	94

APPENDIX 5

The SilviScan-2 latewood averages of density, MFA and MOE for individual samples of growth ring 7.

Sample number	Check type	Avg density (kg/m^3)	Avg MFA (degrees)	Avg MOE (GPa)	Sample size
2A	severe	**18.80208** ±0.887457	**11.61278** ±1.181982	**11.61278** ±1.181982	21
2B	severe	**24.32667** ±0.345491	**8.625033** ±0.946901	**8.625033** ±0.946901	65
3A	severe	**12.58** ±0	**14.2932** ±5.82E-07	**14.2932** ±5.82E-07	59
3B	severe	**22.7** ±0	**9.07377** 0	**9.07377** 0	65
4A	moderate	**13.12** ±0	**7.6904** 0	**7.6904** 0	40
4B	moderate	**11.43686** ±0.114456	**15.2534** ±0.756685	**15.2534** ±0.756685	69
5A	moderate	**20.34** ±0	**10.3419** 0	**10.3419** 0	48
5B	moderate	**17.97463** ±0.327959	**11.19775** ±0.118664	**11.19775** ±0.118664	53
6A	moderate	**16.58** ±0	**11.4973** ±3.37E-07	**11.4973** ±3.37E-07	56
6B	moderate	**16.69** ±0	**11.371** ±2.62E-07	**11.371** ±2.62E-07	53
7A	moderate	**12.24** ±0	**7.3644** ±2.6E-07	**7.3644** ±2.6E-07	27
7B	moderate	**12.02** ±0	**10.3629** 0	**10.3629** 0	26
8A	moderate	**21.2** ±0	**6.79087** 0	**6.79087** 0	26
8B	moderate	**18.88** ±0	**7.58425** 0	**7.58425** 0	31
9A	moderate	**24.64** ±0	**7.17409** 0	**7.17409** 0	43
9B	moderate	**20.3963** ±0.52655	**6.860407** ±0.755668	**6.860407** ±0.755668	26

10A	moderate	**12.472** ±0.386423	**14.785** ±0.710656	**14.785** ±0.710656	79
10B	moderate	**13.88765** ±1.670773	**12.24788** ±1.817655	**12.24788** ±1.817655	50
11A	non check	**15.34** 0	**12.4491** 0	**12.4491** 0	82
11B	non check	**18.85** 0	**10.8193** 0	**10.8193** 0	50
12A	non check	**19.71037** ±0.022161	**10.08781** ±0.559437	**10.08781** ±0.559437	80
12B	non check	**20.30397** ±0.06163	**10.2384** 0.554948	**10.2384** 0.554948	77
13A	non check	**22.48** 0	**8.26161** 0	**8.26161** 0	68
13B	non check	**24.87** 0	**8.17218** 0	**8.17218** 0	70

APPENDIX 6

Averages of the earlywood obtained from the density calculations and modified Fullams micro-test stage for the individual samples.

sample	check	avg den	avg moe	sample size
1	severe	**534.9835** ±208.2721	**4.97E-09** ±4.45E-09	20
2	severe	**451.9225** ±134.3405	**1.67E-08** ±2.44E-08	19
3	severe	**382.6245** ±49.15581	**4.7E-09** ±3.95E-09	11
4	moderate	**352.4225** ±82.06116	**9.11E-09** ±7.41E-09	20
5	moderate	**386.9481** ±125.2933	**7.89E-09** ±4.63E-09	20
6	moderate	**416.9057** ±86.42573	**1.14E-08** ±4.89E-09	20
7	moderate	**310.1932** ±50.21772	**9.85E-09** ±2.9E-09	20
8	moderate	**397.4663** ±72.51259	**1.07E-08** ±5.32E-09	20
9	moderate	**300.2572** ±74.55548	**1.31E-08** ±5.15E-09	20
10	non check	**488.198** ±95.35808	**1.24E-08** ±7.3E-09	20
11	non check	**405.0017** ±65.52932	**1.3E-08** ±5.52E-09	20
12	non check	**389.7471** ±59.41419	**1.41E-08** ±8.6E-09	20
13	non check	**453.1918** ±97.24207	**1.92E-08** ±4.91E-09	18

APPENDIX 7

Averages of the latewood readings obtained from the density calculations and modified Fullams micro-test stage for the individual samples.

sample	check	Avg DENSITY	avg MOE	sample size
1	severe	**638.7698** 125.5416	**1.91E-08** 1.34E-08	18
2	severe	**524.2745** 106.6171	**1.6E-08** 6.73E-09	20
3	severe	**608.2663** 68.52243	**6.99E-09** 7.46E-09	12
4	moderate	**533.7346** 72.10767	**1.63E-08** 8.48E-09	16
5	moderate	**565.3016** 74.32932	**1.84E-08** 1.08E-08	20
6	moderate	**450.3084** 143.5284	**1.69E-08** 8.98E-09	20
7	moderate	**490.2148** 96.77599	**1.45E-08** 7.74E-09	13
8	moderate	**574.4659** 146.5322	**1.5E-08** 1.19E-08	20
9	moderate	**503.6468** 128.2992	**1.24E-08** 9.09E-09	20
10	non check	**621.278** 148.0955	**1.67E-08** 7.73E-09	20
11	non check	**608.1195** 127.9632	**1.53E-08** 1.25E-08	20
12	non check	**734.3381** 212.5373	**2.72E-08** 1.32E-08	20
13	non check	**602.3371** 215.536	**2.15E-08** 1.14E-08	12

APPENDIX 8

The density data analysis of earlywood and latewood data of checked and non-checked wood samples as obtained from the SilviScan-2 and calculated density.

Calculated density			Density as per SilviScan-2		
check	Avg density	sample size	check	Avg density	sample size
severe	**424.5678** ±112.1499	30	severe	**369.9032** ±30.90433	490
moderate	**360.6988** ±93.97911	119	moderate	**356.7657** ±36.38329	1369
no check	**433.5434** ±89.22346	77	no check	**396.7016** ±25.87288	860

TABLE 5: *Density of severe, moderate and non-checked earlywood as per SilviScan-2 and calculated density (kg/m^3) at 40% RH and 20°C. The values obtained from the two methods are different. However, the trends between the checked and non-checked wood are same.* ***The checked wood showed lower density values as compared to the non-checked wood.***

Calculated density			Density as per SilviScan-2		
check	Avg density	sample size	check	Avg density	sample size
severe	**586** ±116.7	50	severe	**581** ±106.6	210
moderate	**521** ±118.6	109	moderate	**559** ±100.9	498
no check	**645** ±172.4	72	no check	**571** ±128.7	418

TABLE 6: *Density of severe, moderate and non-checked latewood as per SilviScan-2 and calculated density (kg/m^3) at 40% RH and 20°C. The values obtained from the two methods are different, however,close to one another. There seems to not too much of difference between the density of the checked and non-checked wood.* ***As per SilviScan-2 in fact the density for severely checked wood is higher than non-checked wood. Whereas the checked wood showed lower density values as compared to the non-checked wood latewood in the calculated density data.***

APPENDIX 9

The MFA data analysis of earlywood and latewood data of checked and non-checked wood samples as obtained from the SilviScan-2 and X-ray diffraction.

X- Ray diffraction data of MFA		
check	Avg MFA (degrees)	sample size
severe	**41.2** ±2.588436	5
moderate	**34** ±2	5
non check	**27.8** ±4.494441	5

TABLE 7: *Earlywood MFA data analysis obtained from X-Ray diffraction.* ***As per X-Ray diffraction technique the MFA of earlywood was higher in checked wood than the non-checked wood****.*

SilviScan-2 data of MFA		
check	Avg MFA (degrees)	sample size
severe	**18.38776** ±4.010732	490
moderate	**18.43472** ±4.118459	1369
non check	**19.14137** ±2.935387	860

Table 8: *Earlywood MFA data analysis obtained from SilviScan-2. The MFA as per SilviScan-2 data seems to be lower than X-ray diffraction data.* ***The average MFA values of the checked wood were lower than those compared to the non-checked wood.***

X- Ray diffraction data of MFA		
check	Avg MFA (degrees)	sample size
severe	**40.5** ±2.828427	2
moderate	**33.85714** ±4.059087	7
non check	**22** 0	1

Table 9: *Latewood MFA data analysis obtained from X-Ray diffraction.* ***As per X-Ray diffraction technique the MFA of latewood was higher in checked wood than the non-checked wood****.*

SilviScan-2 data of MFA		
check	Avg MFA (degrees)	sample size
severe	**19.7436** ±4.433577	210
moderate	**16.92312** ±4.009002	498
non check	**18.50663** ±4.069792	418

Table 10: *Latewood MFA data analysis obtained from SilviScan-2. The MFA as per SilviScan-2 data seems to be lower than X-ray diffraction data.* ***The average MFA values of the severely checked wood higher than compared to the non-checked wood, however, moderately checked wood displayed the lowest average MFA.***

check	Avg crystallinity of cellulose	cellulose intensity	Sample size
severe	**0.3245** ±0.067175	**113350** ±29627.77	2
moderate	**0.450714** ±0.056154	**308400** ±20117.9	7
non check	**0.6555** 0	**291900** 0	1

Table 11: *X- ray diffraction data on cellulose crystallinity and intensity in checked and non-checked latewood.* ***The cellulose had lower level of crystallinity and intensity in checked wood as compared to non-checked wood.***

APPENDIX 10

The MOE data analysis of earlywood and latewood data of checked and non-checked wood samples as obtained from the SilviScan-2 and modified Fullams-micro test stage.

MOE data from SilviScan-2			MOE data from modified Fullam micro- test stage		
check	Avg MOE (GPa)	sample size	check	Avg MOE(GPa)	sample size
severe	**9.222421** ±1.593044	490	severe	**9.11E-09** ±8.76E-09	31
moderate	**8.102587** ±2.200745	1369	moderate	**1.03E-08** ±5.37E-09	119
non check	**9.206226** ±1.423378	860	non check	**1.46E-08** ±7.1E-09	77

Table 12: *The analysis of the earlywood MOE data obtained from Silcisvan-2 and the modified Fullams micro-test stage.* ***There is no clear trend emerging from the SilviScan-2 data but from the Fullams data we can see that the checked wood has lower MOE compared to the non-checked wood****.*

MOE data from SilviScan-2			MOE data from modified Fullam micro- test stage		
check	Avg MOE (GPa)	sample size	check	Avg MOE(GPa)	sample size
severe	10.84028 ±2.34451	210	severe	**1.49E-08** ±1.07E-08	50
moderate	10.15058 ±2.727643	498	moderate	**1.56E-08** ±9.74E-09	109
non check	10.9193 ±2.249711	418	non check	**1.99E-08** ±1.17E-08	72

Table 12: *The analysis of the latewood MOE data obtained from Silcisvan-2 and the modified Fullams micro-test stage.* ***There is not much difference in the MOE values between the checked and non-checked wood in the data obtained from both SilviScan-2 and Fullams data, however, we do see that the checked wood have lower MOE than the non-checked wood.***

APPENDIX 11

The measurements of the growth rings and the checks in the checked oven-dreid discs of radiata pine.

Sample no	severity of check	proportion of the tangential dimension of the disc that was checked	check width range (cm)	growth ring width range (cm) checked zone	growth ring width range (cm) non checked zone
1	severe	100.00	0.4- 0.3	2.5 -1.2	0.5 - 0.4
2	severe	100.00	0.3	1.2 -1	0.5 - 0.4
3	severe	100.00	0.3	1 - 0.8	0.5 - 0.4
4	moderate	71.43	0.1	1.2 - 0.8	0.5 - 0.4
5	moderate	83.33	0.1	1.5 - 1.2	0.5 - 0.4
6	moderate	60.00	0.05 - 0.02	1.5 - 1	0.5 - 0.4
7	moderate	85.71	0.02- 0.01	1.5 - 1.3	0.5 - 0.4
8	moderate	32.43	0.02 - 0.01	1.2 - 1	0.5 - 0.4
9	moderate	46.88	0.02 -0.02	1.5 - 1.2	0.5 - 0.4
10	no check	0.00	0	0.7 - 0.8	0.5 - 0.4
11	no check	0.00	0	0.7 - 1	0.5 - 0.4
12	no check	0.00	0	0.9 - 1	0.5 - 0.4
13	no check	0.00	0	1	0.5 - 0.4

APPENDIX 12

The data below is the average range of the type of cells observed with the help of light microscopy in the growth rings of the SilviScan-2 samples when the wood was dry and after the same samples were saturated with water.

Sample	Sample type	Ring number	Range of cell collapse type(dry)	Range of cell collapse type(wet)	If intra-ring check present in the sample
1A	severe	1	bad collapse	bad collapse	no check
1A	severe	2	bad collapse	bad collapse	adjacent to check
1A	severe	3	bad collapse	bad collapse	no check
1A	severe	4	bad collapse	bad collapse	adjacent to check
1A	severe	5	bad collapse	bad collapse	no check
1A	severe	6	mild collapse	least collapse	no check
1A	severe	7	mild collapse	least collapse	no check
1B	severe	1	bad collapse	bad collapse	no check
1B	severe	2	bad collapse	bad collapse	check
1B	severe	3	bad collapse	bad collapse	3 checks
1B	severe	4	bad collapse	mild collapse	check
1B	severe	5	bad collapse	mild collapse	check
1B	severe	6	mild collapse	mild collapse	no check
1B	severe	7	bad collapse	mild collapse	check
2A	severe	4	mild collapse	mild collapse	no check
2A	severe	5	bad collapse	mild collapse	no check
2A	severe	6	mild collapse	least collapse	check
2A	severe	7	least collapse	least collapse	check
2A	severe	8	mild collapse	mild collapse	no check
2A	severe	9	east collapse	least collapse	no check
2A	severe	10	mild collapse	mild collapse	no check
2A	severe	11	no collapse	no collapse	no check
2A	severe	12	no collapse	no collapse	no check
2A	severe	13	no collapse	no collapse	no check
2A	severe	14	no collapse	no collapse	no check
2A	severe	15	no collapse	no collapse	no check
2B	severe	1	least collapse	no collapse	no check
2B	severe	2	mild collapse	no collapse	check
2B	severe	3	bad collapse	least collapse	2 checks
2B	severe	4	mild collapse	least collapse	check
2B	severe	5	bad collapse	mild collapse	check
2B	severe	6	least collapse	least collapse	adjacent to check
2B	severe	7	mild collapse	least collapse	2 checks
2B	severe	8	mild collapse	least collapse	2 checks
2B	severe	9	least collapse	least collapse	check
2B	severe	10	no collapse	no collapse	no check
3A	severe	1	least collapse	no collapse	no check

3A	severe	2	no collapse	no collapse	no check
3A	severe	3	mild collapse	least collapse	adjacent to check
3A	severe	4	bad collapse	least collapse	check
3A	severe	5	mild collapse	mild collapse	adjacent to check
3A	severe	6	mild collapse	least collapse	no check
3A	severe	7	mild collapse	least collapse	no check
3A	severe	8	least collapse	least collapse	no check
3B	severe	1	least collapse	least collapse	no check
3B	severe	2	no collapse	no collapse	no check
3B	severe	3	least collapse	least collapse	no check
3B	severe	4	least collapse	least collapse	no check
3B	severe	5	mild collapse	least collapse	no check
3B	severe	6	least collapse	no collapse	no check
3B	severe	7	least collapse	no collapse	no check
3B	severe	8	no collapse	no collapse	no check
3B	severe	9	no collapse	no collapse	no check
3B	severe	10	no collapse	no collapse	no check
3B	severe	11	no collapse	no collapse	no check
3B	severe	12	no collapse	no collapse	no check
3B	severe	13	no collapse	no collapse	no check
3B	severe	14	no collapse	no collapse	no check
4A	moderate	1	no collapse	no collapse	no check
4A	moderate	2	least collapse	least collapse	no check
4A	moderate	3	no collapse	no collapse	no check
4A	moderate	4	no collapse	no collapse	no check
4A	moderate	5	no collapse	no collapse	no check
4A	moderate	6	no collapse	no collapse	no check
4A	moderate	7	no collapse	no collapse	no check
4A	moderate	8	least collapse	no collapse	no check
4A	moderate	9	least collapse	no collapse	no check
4A	moderate	10	least collapse	no collapse	no check
4A	moderate	11	no collapse	no collapse	no check
4A	moderate	12	no collapse	no collapse	no check
4A	moderate	13	no collapse	no collapse	no check
4A	moderate	14	no collapse	no collapse	no check
4A	moderate	15	no collapse	no collapse	no check
4A	moderate	16	no collapse	no collapse	no check
4A	moderate	17	no collapse	no collapse	no check
4A	moderate	18	no collapse	no collapse	no check
4B	moderate	1	no collapse	no collapse	no check
4B	moderate	2	no collapse	no collapse	no check
4B	moderate	3	no collapse	no collapse	check
4B	moderate	4	no collapse	no collapse	check
4B	moderate	5	mild collapse	least collapse	check
4B	moderate	6	no collapse	no collapse	check
4B	moderate	7	least collapse	no collapse	no check

4B	moderate	8	no collapse	no collapse	no check
4B	moderate	9	no collapse	no collapse	no check
4B	moderate	10	least collapse	least collapse	no check
4B	moderate	11	least collapse	no collapse	no check
4B	moderate	12	least collapse	least collapse	no check
4B	moderate	13	least collapse	no collapse	no check
5A	moderate	1	no collapse	no collapse	no check
5A	moderate	2	mild collapse	no collapse	no check
5A	moderate	3	no collapse	no collapse	no check
5A	moderate	4	mild collapse	no collapse	no check
5A	moderate	5	least collapse	no collapse	no check
5A	moderate	6	least collapse	no collapse	no check
5A	moderate	7	least collapse	no collapse	no check
5A	moderate	8	least collapse	no collapse	no check
5A	moderate	9	no collapse	no collapse	no check
5A	moderate	10	no collapse	no collapse	no check
5A	moderate	11	no collapse	no collapse	no check
5B	moderate	1	no collapse	no collapse	no check
5B	moderate	2	least collapse	no collapse	no check
5B	moderate	3	least collapse	no collapse	no check
5B	moderate	4	no collapse	no collapse	no check
5B	moderate	5	no collapse	no collapse	no check
5B	moderate	6	least collapse	no collapse	no check
5B	moderate	7	least collapse	no collapse	no check
5B	moderate	8	least collapse	no collapse	no check
5B	moderate	9	no collapse	no collapse	no check
5B	moderate	10	no collapse	no collapse	no check
5B	moderate	11	no collapse	no collapse	no check
5B	moderate	12	no collapse	no collapse	no check
5B	moderate	13	no collapse	no collapse	no check
5B	moderate	14	no collapse	no collapse	no check
5B	moderate	15	no collapse	no collapse	no check
5B	moderate	16	no collapse	no collapse	no check
6A	moderate	1		no collapse	no check
6A	moderate	2	mild collapse	no collapse	no check
6A	moderate	3	mild collapse	no collapse	check
6A	moderate	4	no collapse	no collapse	check
6A	moderate	5	no collapse	no collapse	no check
6A	moderate	6	mild collapse	no collapse	no check
6A	moderate	7	least collapse	no collapse	no check
6A	moderate	8	no collapse	no collapse	no check
6A	moderate	9	no collapse	no collapse	no check
6A	moderate	10	least collapse	no collapse	no check
6A	moderate	11	no collapse	no collapse	no check
6A	moderate	12	no collapse	no collapse	no check
6B	moderate	4	no collapse	no collapse	no check

6B	moderate	5	least collapse	no collapse	no check
6B	moderate	6	no collapse	no collapse	no check
6B	moderate	7	least collapse	no collapse	no check
6B	moderate	8	no collapse	no collapse	no check
6B	moderate	9	least collapse	no collapse	no check
6B	moderate	10	least collapse	least collapse	no check
6B	moderate	11	no collapse	no collapse	no check
6B	moderate	12	no collapse	no collapse	no check
6B	moderate	13	no collapse	no collapse	no check
6B	moderate	14	no collapse	no collapse	no check
6B	moderate	15	no collapse	no collapse	no check
6B	moderate	16	no collapse	no collapse	no check
7A	moderate	8	no collapse	no collapse	no check
7A	moderate	9	no collapse	no collapse	no check
7A	moderate	10	no collapse	no collapse	no check
7A	moderate	11	no collapse	no collapse	no check
7A	moderate	12	no collapse	no collapse	no check
7A	moderate	13	least collapse	no collapse	no check
7A	moderate	14	no collapse	no collapse	no check
7A	moderate	15	no collapse	no collapse	no check
7A	moderate	16	no collapse	no collapse	no check
7A	moderate	17	no collapse	no collapse	no check
7A	moderate	18	no collapse	no collapse	no check
7B	moderate	8	no collapse	no collapse	no check
7B	moderate	9	least collapse	no collapse	no check
7B	moderate	10	no collapse	no collapse	no check
7B	moderate	11	no collapse	no collapse	no check
7B	moderate	12	no collapse	no collapse	no check
7B	moderate	13	no collapse	no collapse	no check
7B	moderate	14	no collapse	no collapse	no check
7B	moderate	15	no collapse	no collapse	no check
7B	moderate	16	no collapse	no collapse	no check
7B	moderate	17	no collapse	no collapse	no check
7B	moderate	18	no collapse	no collapse	no check
8A	moderate	1	no collapse	no collapse	no check
8A	moderate	2	mild collapse	no collapse	no check
8A	moderate	3	mild collapse	no collapse	no check
8A	moderate	4	no collapse	no collapse	no check
8A	moderate	5	mild collapse	no collapse	no check
8A	moderate	6	no collapse	no collapse	no check
8A	moderate	7	least collapse	no collapse	no check
8A	moderate	8	least collapse	no collapse	no check
8A	moderate	9	least collapse	no collapse	no check
8A	moderate	10	no collapse	no collapse	no check
8A	moderate	11	no collapse	no collapse	no check
8A	moderate	12	no collapse	no collapse	no check

8A	moderate	13	no collapse	no collapse	no check
8A	moderate	14	least collapse	no collapse	no check
8A	moderate	15	no collapse	no collapse	no check
8A	moderate	16	no collapse	no collapse	no check
8B	moderate	1	no collapse	no collapse	no check
8B	moderate	2	no collapse	no collapse	no check
8B	moderate	3	mild collapse	least collapse	no check
8B	moderate	4	mild collapse	mild collapse	no check
8B	moderate	5	mild collapse	least collapse	check
8B	moderate	6	least collapse	mild collapse	no check
8B	moderate	7	least collapse	least collapse	no check
8B	moderate	8	no collapse	least collapse	no check
8B	moderate	9	no collapse	mild collapse	no check
8B	moderate	10	least collapse	no collapse	no check
8B	moderate	11	no collapse	no collapse	no check
8B	moderate	12	no collapse	no collapse	no check
8B	moderate	13	no collapse	no collapse	no check
8B	moderate	14	least collapse	no collapse	no check
8B	moderate	15	no collapse	no collapse	no check
8B	moderate	16	no collapse	no collapse	no check
9A	moderate	2	least collapse	no collapse	no check
9A	moderate	3	least collapse	no collapse	no check
9A	moderate	4	no collapse	no collapse	no check
9A	moderate	5	mild collapse	no collapse	no check
9A	moderate	6	least collapse	no collapse	no check
9A	moderate	7	least collapse	no collapse	no check
9A	moderate	8	no collapse	no collapse	no check
9A	moderate	9	no collapse	no collapse	no check
9A	moderate	10	no collapse	no collapse	no check
9A	moderate	11	no collapse	no collapse	no check
9A	moderate	12	no collapse	no collapse	no check
9A	moderate	13	no collapse	no collapse	no check
9A	moderate	14	no collapse	no collapse	no check
9A	moderate	15	no collapse	no collapse	no check
9A	moderate	16	no collapse	no collapse	no check
9B	moderate	2	no collapse	no collapse	no check
9B	moderate	3	no collapse	no collapse	no check
9B	moderate	4	no collapse	no collapse	no check
9B	moderate	5	no collapse	no collapse	no check
9B	moderate	6	no collapse	least collapse	no check
9B	moderate	7	mild collapse	least collapse	check
9B	moderate	8	least collapse	least collapse	check
9B	moderate	9	least collapse	no collapse	check
9B	moderate	10	no collapse	no collapse	no check
9B	moderate	11	no collapse	no collapse	no check
9B	moderate	12	no collapse	no collapse	no check